

**Technologies for tissue preservation:
the role of endogenous and
exogenous antioxidants in preserving
tissue function in chinook salmon,
*Oncorhynchus tshawytscha***

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requirements for the Degree
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by

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List of abbreviations

AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
DHA	Dehydroascorbate
DNPH	2,4-dinitrophenyl hydrazine
EDTA	Ethylendiaminetetraacetic acid
FADH ₂	Reduced flavin adenine dinucleotide
GSH	Glutathione
GSH-px	Glutathione peroxidase
GSSG	Glutathione disulfide
HPLC	High performance liquid chromatography
IMP	Inosine monophosphate
Ino	Inosine
LDH	Lactate dehydrogenase
MBB	Monobromobimane
MDA	Malondialdehyde
NAD ⁺	β -nicotinamide adenine dinucleotide
NADH	Reduced β -nicotinamide adenine dinucleotide
NADP	β -nicotinamide adenine dinucleotide phosphate
NADPH	Reduced β -nicotinamide adenine dinucleotide phosphate
OPA	<i>O</i> -phthaldialdehyde
PCA	Perchloric acid
P _i	Inorganic phosphate
PVP	Polyvinylpyrrolidone
ROOH	Hydroperoxide
SOD	Superoxide dismutase
TBA	2-thiobarbituric acid

TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

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Abstract

The seafood industry is of considerable importance to both the New Zealand and global economies and therefore tissue preservation technologies that increase product quality and/or prolong shelf life have the potential to add significant value. Technologies for maintaining the viability of isolated tissues also have a wide range of other medical and industrial applications. This thesis examines the relationship between metabolic function, oxidation and cell death and the resulting stability of the non-viable tissues during long term storage in chinook salmon (*Oncorhynchus tshawytscha*) red and white skeletal muscle tissue. This research also looks at the role of the aquatic anaesthetic AQUI-S™, in which the active ingredient is isoeugenol (a lipid soluble antioxidant), and other antioxidant compounds in preserving metabolic function in viable tissues and tissue stability in non-viable tissues.

Perfusion of salmon tails at 15°C over 5 or 10 hours with oxygen saturated saline resulted in significant increases in protein and lipid oxidation (protein carbonyl and TBARS concentrations respectively) in the red muscle, but not the white muscle. The introduction of ascorbic acid and uric acid into the saline did not reduce the oxidation in the red muscle despite significantly increasing their respective concentrations in the tissue. This indicates the difficulties associated with attempting to extend tissue viability by delivering free oxygen to the tissue and also highlights the difference in susceptibility of the two muscle types to oxidation.

Tail fillets from salmon harvested in both rested and exhausted physiological states using AQUI-S™, and fillets from exhausted salmon harvested without AQUI-S™, were exposed to air at 15°C for up to 96 hours. Protein carbonyls increased in a roughly linear fashion over the entire 96 hours in all three groups. Both lipid peroxides (TBARS) and uric acid concentrations began to increase in the exhausted group after 30 hours. In contrast, no significant increases in lipid peroxides or uric acid was seen in the fillets from either group harvested using AQUI-S™. Vitamin E concentrations reduced slowly but did not change significantly despite the oxidation that was evident in the tissue. These processes also occurred in salmon tail fillets during storage at 6°C.

The measurement of ATP related compounds provides an effective indicator of both the metabolic state of the tissue post-harvest and the quality. The breakdown of these compounds is also associated with the production of ammonia and hydrogen peroxide. Fresh rested salmon fillets had high concentrations of ATP and creatine phosphate, which

were both depleted after 12 hours storage at 15°C. This indicates that cell viability lasted a number of hours following harvesting. These metabolites were depleted in exhausted fillets and metabolic potential appeared to be immediately compromised. The concentration of the taste enhancing compound IMP was significantly reduced in fresh rested tissue, but increased during storage, and was significantly higher than in exhausted tissues following 12 hours of storage at 15°C. This indicates that some properties of rested tissues may improve with limited storage times. The accumulation of uric acid – the metabolic end point for ATP related compounds - was also significantly reduced in rested tissue and increases in K-value were slowed.

AQUI-STM showed an ability to preserve tissue function through its anaesthetic action allowing tissue to be harvested in a rested state, and to reduce late stage lipid oxidation in stored salmon tail fillets. The antioxidant action of isoeugenol in salmon fillets may be mediated through its ability to chelate transition metals released during tissue degradation.

This research shows that during reperfusion and during fillet storage there is a significant level of oxidative stress, which needs to be minimized while maintaining basic tissue metabolism to prolong tissue and cellular viability. The development of future technologies to preserve tissue viability may depend on the development of a synthetic oxygen carrying compound with properties similar to red blood cells. This may allow more control over oxygen delivery, potentially reducing the oxidative stress associated with high concentrations of free oxygen in solution. However, preserving cell viability will also require the maintenance of endogenous antioxidant function and there is also the potential to use iron chelating compounds including plant derived flavonoids to preserve non-viable tissues. Future research in these areas is necessary.

1 - Introduction

The seafood industry in New Zealand is well established and contributed \$1 351 million to export earnings in 2006, with \$28 million coming directly from farmed chinook salmon (*Oncorhynchus tshawytscha*) (The New Zealand Seafood Industry Council Ltd. website 2007). Although New Zealand's production of salmon (7000 tonnes in 2005) is a small component of worldwide production (approximately 1.4 million tonnes annually), chinook salmon fill a niche high quality market due to their attractive flesh colour and high oil content (NIWA website 2007). Therefore technologies and knowledge that add value, not only to the salmon farming industry but to the seafood industry as a whole have the potential to be of great benefit to the New Zealand economy.

Two important factors contributing to the value of the seafood industry are product quality and shelf life, which are linked and heavily dependent on the natural physiology and biochemistry of the animal. In the case of animals that are farmed specifically for human consumption there are a number of factors, many of which are human influenced, that contribute to the quality of the end product. These include water quality, diet and environmental factors that influence behaviour. Environmental factors that cause stress have been shown to negatively impact growth and immune function (Wendelaar Bonga 1997; Einarsdottir et al. 2000; Ashley 2007). One of the major stress causing events in aquaculture is human handling associated with animal husbandry practices and harvesting (Davis and Schreck 1997; Sigholt et al. 1997; Ashley 2007). During harvesting, the biochemical and physiological changes induced in the muscle tissue by the exhaustive struggling associated with the crowding, chasing and air exposure inherent in many harvesting procedures are carried through into the final product, which has been shown to affect quality (Sigholt et al. 1997; Bagni et al. 2007; Bosworth et al. 2007; Ribas et al. 2007). The development of anaesthetics such as AQUI-STM for use in the aquaculture industry has allowed the stressful events associated with human handling to be much better managed and many of the negative impacts significantly reduced. It has also allowed characterization of some of the physiological and biochemical properties of rested fish tissues, both fresh and during storage and has shown that they are significantly different from exhausted tissues (Jerrett and Holland 1998; Jerrett et al. 2000; Black 2002; Bosworth et al. 2007). In particular the preservation of large quantities of energy carrying metabolites in the white muscle tissue such as ATP and creatine phosphate, and the associated low levels of metabolic byproducts such as lactate, mean that the cells in the

white muscle tissue can remain viable for a considerable period of time post-mortem. Previous work has shown that this period of cell viability can be extended through manipulating the storage conditions of the ischemic tissue (Black 2002), or through perfusion (Janssen 2003). Both techniques aim to help maintain cellular homeostasis and deliver compounds such as oxygen that are essential for normal cell function. Although these techniques proved successful for a period of time, cell viability was still eventually lost. As oxygen is linked to the production of free radicals through the mitochondrial electron transport chain (Halliwell and Gutteridge 1999; Cadenas and Davies 2000; Sachdev and Davies 2008), oxidative damage to important cellular components was put forward as a potential explanation for this loss of cell viability (Black 2002; Janssen 2003). This thesis explores the relationship between metabolic state and oxidative damage in salmon skeletal muscle and examines the potential for extending cell viability and/or preserving harvested tissue using compounds that have antioxidant properties.

1.1 Storage techniques for viable and non-viable tissues

1.1.1 Temperature

Chilling of tissues is the most widely used preservation technique. When used appropriately, chilling can extend cell viability, as in transplant organs (Vigues et al. 1993; Stoica et al. 2001), or can be used to prevent spoilage and extend the shelf life of food products. Ice storage in particular is widely used during processing and storage of seafood to preserve harvested tissues (Undeland et al. 1999; Espe et al. 2004; Kiessling et al. 2004; Aubourg et al. 2005; Aubourg et al. 2005a).

The effectiveness of reduced temperatures for tissue storage is due to the reduced thermal kinetic energy of the molecules in the tissue. This slows reaction rates by reducing the number of chemical interactions that exceed the activation energy required for reactions to proceed (Withers 1992). This prevents many normal biochemical reactions from occurring in viable tissues. As the storage of viable tissues generally involves ischemia (Stoica et al. 2001), the requirements of the tissue are not met for normal tissue function and without metabolic suppression this will rapidly lead to cell death. Storage techniques for viable tissues remain limited, with the safe ischemic period for transplant hearts remaining at 4 hours (Stoica et al. 2001).

Refrigeration is effective in prolonging the shelf life of food products, reducing the rate of undesirable chemical reactions, such as those resulting in lipid oxidation (Orlien et al. 2006). This is particularly important for products like salmon fillets as they contain high proportions of unsaturated lipids that are susceptible to oxidation (Kennedy et al. 2005; Kiessling et al. 2005; Nanton et al. 2007). Refrigeration is also effective in inhibiting the growth of bacteria, which is also associated with the spoilage of seafood (Lyhs et al. 2001; Rasmussen et al. 2002; Robson et al. 2007).

1.1.2 Perfusion

Perfusion of isolated tissues as a scientific technique has been utilized for a number of decades (Satchell 1964; Shuttleworth 1972; Moen et al. 1981; Driedzic et al. 1982; Part et al. 1982; Rothwell and Forster 2005). The isolation of tissues prevents interactions with other tissues such as internal organs, allowing better experimental control. This allows tissue specific properties and/or the interaction of compounds of interest with specific tissues to be more accurately investigated (Moen et al. 1981; Plin et al. 2005; Rothwell and Forster 2005). For this to be effective the perfusion must maintain the normal behaviour of the tissue to the greatest extent possible. There are many different variations of physiological saline, however most firstly address colloid osmotic pressure and ionic balance (Skrzypiec-Spring et al. 2007). Preparations that contain complete physiological structures must deliver the saline by pumping it through the intact vasculature. In this case maintaining an appropriate hydrostatic pressure is important for adequate perfusion of the tissue and to avoid the accumulation of interstitial fluid, known as oedema.

Practical uses for perfusion outside of research are currently restricted to medical tissue preservation, although these preparations generally do not aim to maintain the normal metabolic function of the organ. In fact the low oxygen carrying capacity of salines may prevent them from doing so, or lead to abnormally high perfusion rates (Skrzypiec-Spring et al. 2007). Instead the aim is to stabilize the tissue and prevent cold and reperfusion damage (Xu et al. 2005; Bessems et al. 2005a; Bessems et al. 2005b; Bessems et al. 2006; Rauen et al. 2007). The closest application of perfusion principles to seafood products so far has been the washing or soaking of fillets in water both with and without compounds that may improve fillet quality such as ascorbic acid (Richards et al. 1998; Chaijan et al. 2005).

1.1.3 Hypometabolism

Although the induction of a sustainable hypometabolic state is not currently possible for the long term storage of viable tissues, it is of considerable interest due to its common use in estivating and anoxia tolerant animals (Storey 2002; Milton and Prentice 2007). The processes allowing for a hypometabolic state are complex and tightly regulated. The animal must firstly cope with the initial transition to a hypometabolic state that typically involves decreasing the demand for ATP to between 10 and 30% of normal resting metabolic rate. This involves strategies such as reducing ion leakage across membranes and arresting ion channels (Milton and Prentice 2007). A more prolonged hypometabolic state must then be maintained during which energy supply and demand are closely matched to maintain ATP levels. Finally there is the recovery phase during which the metabolism is elevated back to resting (Milton and Prentice 2007). Recovery has been shown to be associated with the over production of damage causing oxygen centred radicals, and may require adaptation of the endogenous antioxidant systems to prevent tissue damage (Ramos-Vasconcelos and Hermes-Lima 2003).

Developments in inducing and maintaining a hypometabolic state in tissues would greatly contribute to fields such as organ transplantation. These principles may also prove useful in improving the quality and prolonging the shelf life of meat and seafood products. However it remains to be seen whether the cellular and physiological processes required for hypometabolism can be initiated in tissues that do not normally experience these events.

1.2 Skeletal muscle physiology of teleost fish

Fish muscle, much like other vertebrates, is composed of contractile proteins, lipids (from cell membranes, adipocytes and intracellular lipid droplets) and connective tissue, all of which affect the properties of the meat. Unlike higher vertebrates however, the fibre types in fish muscle are highly compartmentalized and 90-95% of the total mass is glycolytic anaerobic white muscle (Kiessling et al. 2006). Although there is some overlap in the use of the red and white muscle fibres, the white muscle is used primarily for high speed burst swimming associated with prey capture and escape responses. The white muscle is poorly vascularized and functions as an almost closed system during burst exercise (Weber and Haman 1996). The fibres are dominated by myofibrils and contain few mitochondria and

relatively small amounts of glycogen (Kiessling et al. 2006; Nanton et al. 2007). Unlike many other fish species, salmonid white muscle contains significant amounts of lipid, predominantly found as triacylglycerol in adipocytes (fat storage cells) in the myosepta (Zhou et al. 1996; Nanton et al. 2007). The myosepta appear as pale bands of connective tissue running through the muscle. Smaller quantities of lipid are associated with the cell membranes and mitochondria (Nanton et al. 2007). Lipids provide a fuel source for aerobic respiration, however, given the paucity of mitochondria and low blood flow to white muscle tissue it is unlikely that aerobic metabolism contributes much to the energy demands of burst exercise. Instead, its contribution is likely to be predominantly for the maintenance of cellular homeostasis and recovery from exercise. Most of the energy required for burst exercise is supplied by the anaerobic breakdown of glycogen (glycolysis) and intracellular stores of creatine phosphate and ATP (Milligan 1996; Kieffer 2000). These stores are limited and exhaustion generally occurs within a few minutes and complete recovery can take a number of hours (Milligan 1996). There is marked heterogeneity in size and metabolic potential in white muscle fibres, with smaller fibres containing far more glycogen and greater aerobic potential (Kiessling et al. 1990). This may allow more efficient recycling of lactate produced during burst exercise and/or allow the recruitment of smaller white fibres during aerobic exercise (Kiessling et al. 2004; Kiessling et al. 2006). Recent work has shown certain fibre types within a working muscle can be preferentially utilized in higher vertebrates (Wakeling et al. 2006).

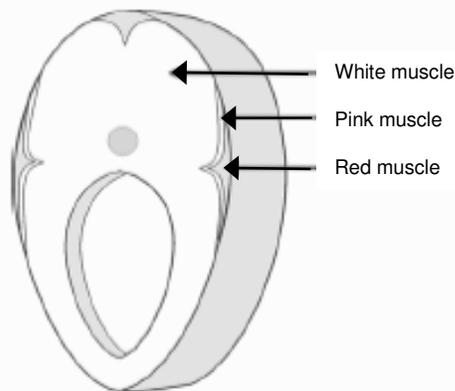


Figure 1.1 Cross section of a teleost fish showing the location of red (highly aerobic, slow twitch), pink (highly aerobic, highly glycolytic, fast twitch) and white (anaerobic, highly glycolytic, fast twitch) muscle fibres. (from Kiessling et al. 2006a)

Red muscle in teleost fish is located in wedge shaped bands below the lateral line. It generally makes up 10% or less of the total skeletal muscle mass and is used for low

velocity sustained swimming. The fibres are small in diameter (25-45 μm) and very well vascularized (Kiessling et al. 2006), receiving approximately $2/3$ of the blood flow to the skeletal muscle in a resting chinook salmon (Janssen 2003). They are densely packed with mitochondria, lipid droplets, glycogen and myoglobin and their metabolism is almost entirely aerobic, with lipids being the primary fuel source and carbohydrates secondary (Kiessling et al. 2006).

Pink fibres are found in many teleost species as a narrow band separating the red and white muscle. Their function and biochemistry appears to fall between that of the red and white muscle. Salmonids however do not have this particular fibre type (Martinez et al. 1993; Kiessling et al. 1995; Kiessling et al. 2006).

1.3 Aerobic and anaerobic energy production

Energy production is essential for life. In skeletal muscle tissue it is required for the maintenance of cellular homeostasis and the contractile properties that allow locomotion. Cellular viability and functionality are therefore intricately linked to the general well being of the energy production pathways within the cell.

The energy produced in working muscles is ultimately derived from the protein, lipid and polysaccharides consumed as food, which are broken down during digestion and transported to the cells where they are used to produce energy. Simple sugars such as glucose are converted to pyruvate via glycolysis in the cell's cytoplasm. This produces a net gain of two molecules of ATP per molecule of glucose. This process is particularly important in anaerobic tissues such as teleost white muscle as it allows the generation of energy in the absence of oxygen (Alberts et al. 1994). During anaerobic metabolism the pyruvate is converted to lactate by lactate dehydrogenase (LDH) allowing the coupled regeneration of NAD^+ , which is essential for glycolysis to be able to continue (Withers 1992). Following exercise the lactate can then be converted back to pyruvate either in situ or following transport to aerobic tissues. In teleost fishes, much of the lactate produced during burst exercise is reprocessed in situ (Milligan 1996).

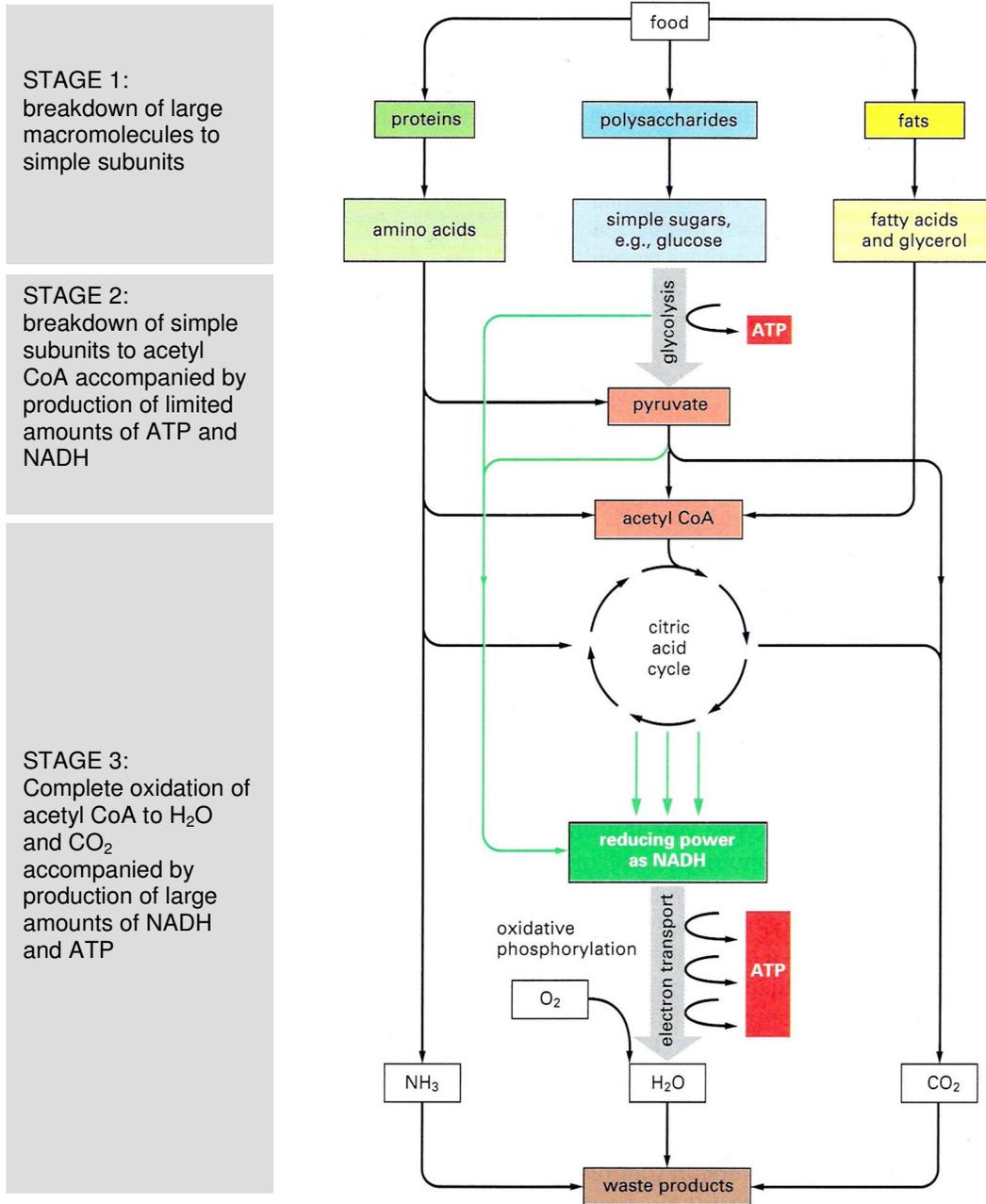


Figure 1.2 Simplified diagram of the three stages of catabolism that lead from food to waste products. The series of reactions produces ATP, which is then used to drive biosynthetic reactions and other energy requiring processes in the cell. (from Alberts et al. 1994)

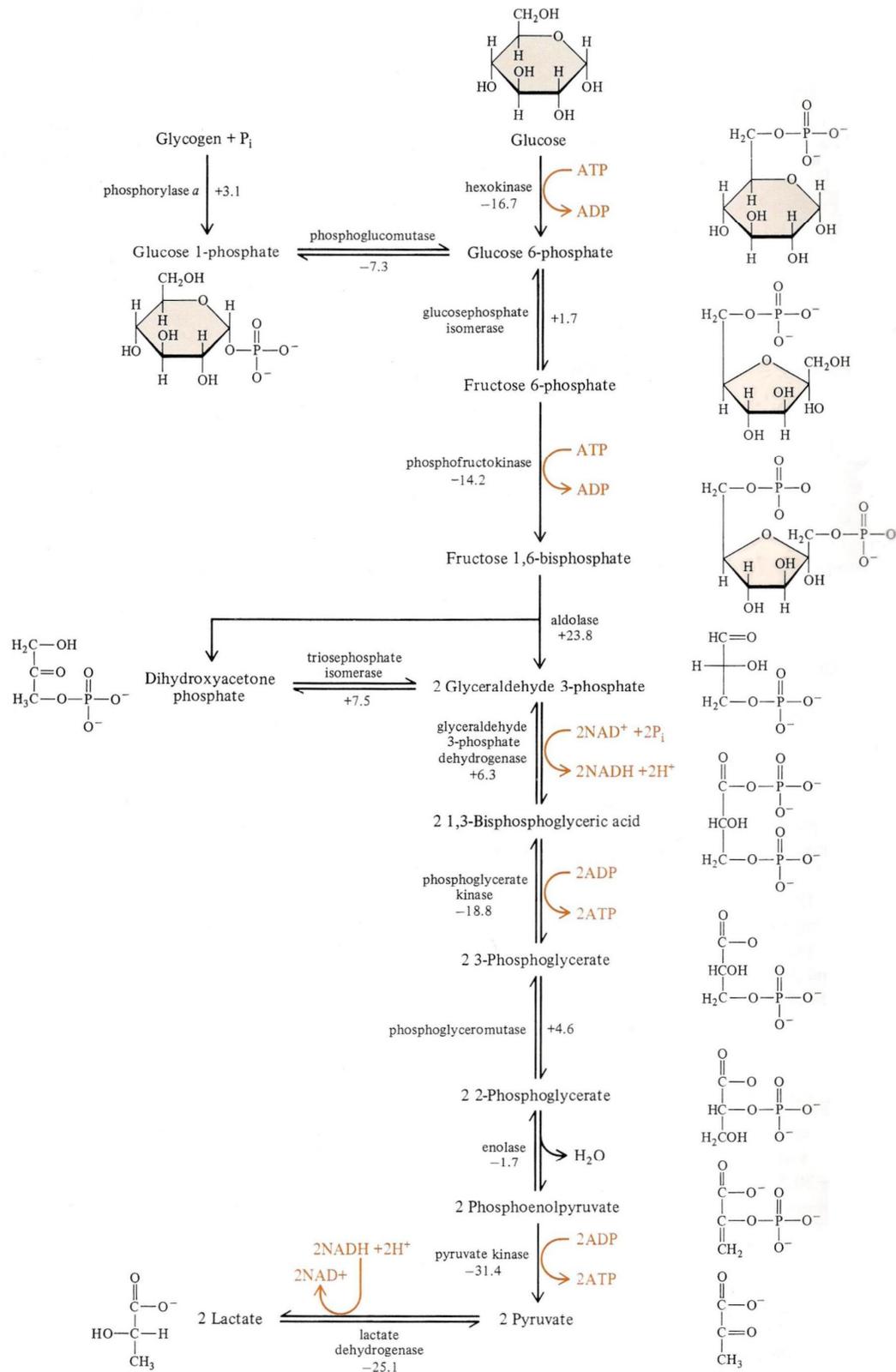


Figure 1.3 Schematic pathway for glycolysis in animal cells showing the individual reactions, Gibbs free energy changes (ΔG° ; kJ mole⁻¹), chemical structures, and overall energetics for glucose and glucosyl subunits. (from Withers 1992)

In the presence of oxygen, amino acids, sugars and fatty acids are broken down completely to form CO₂ and H₂O in the mitochondrion. It is the final stages of oxidative phosphorylation that produce the majority of the metabolic energy, some of which is captured and the rest released as heat. During this process NADH and FADH₂ transfer the electrons they are carrying from the oxidation of food molecules to O₂, producing H₂O. This transfer of electrons occurs on the inner membrane of the mitochondrion. The energy state of the electrons is reduced in a stepwise manner, which drives the transfer of protons across the inner membrane creating an electrochemical proton gradient. This gradient drives the protons through an enzyme complex and causes ATP synthase to add a phosphate group to ADP (Withers 1992; Alberts et al. 1994). The resulting ATP is then transferred to the cytoplasm where it is used predominantly to drive processes important for the maintenance of cellular homeostasis, including driving Na⁺/K⁺ pumps, protein synthesis and protein degradation (Hochachka et al. 1996).

The relative contributions of these two energy generation systems are tissue specific. Teleost white muscle shows a much greater anaerobic potential and a limited aerobic potential compared to red muscle (Kiessling et al. 2006). However there is considerable overlap between the two systems both within individual cells and the animal as a whole. For example, during rest and recovery, teleost white muscle must function almost entirely aerobically (although at a rate far below that achievable by red muscle) to maintain or regain homeostasis. If oxygen is available during burst exercise, aerobic metabolism will continue in the tissue despite the dominance of anaerobic energy production. In the whole animal, there is also overlap in the recruitment of the two fibre types during exercise (Kiessling et al. 2006).

1.4 ATP related compounds and storage properties in skeletal fish muscle

ATP and its related metabolites can be used to provide an indication of the metabolic state of a viable tissue and the freshness of a non-viable tissue during storage. Generally, the breakdown of ATP related compounds takes place in the order ATP → ADP → AMP → inosine monophosphate (IMP) → inosine → hypoxanthine → xanthine → uric acid (Luong et al. 1989; Carsol et al. 1997; Vazquez-Ortiz et al. 1997). The K-value of a tissue is expressed as a percentage and represents the ratio of the ATP breakdown products hypoxanthine and inosine to the total pool of ATP related compounds (Saito et al. 1959;

Ryder 1985). It has been shown to reliably indicate the freshness of seafood and removes many of the issues associated with using a single metabolite (Ryder 1985; Vazquez-Ortiz et al. 1997; Dondero et al. 2004; Aubourg et al. 2005a; Aubourg et al. 2007). IMP concentrations in particular have taste and quality implications as IMP is known to contribute to the umami-taste of fish products (Kawai et al. 2002; Kuda et al. 2007).

The loss of tissue ATP is also involved in the onset of rigor-mortis, which has quality and processing implications for fish products and is affected by pre-harvest stress and exhaustion (Sigholt et al. 1997; Jerrett and Holland 1998; Roth et al. 2006). However, K-values are not able to resolve the onset of rigor-mortis as it remains close to zero until late stage breakdown products of ATP are generated. Individual metabolite measurements may prove more useful for this purpose.

The cycling of NAD^+ and NADH are redox reactions and this cycle has many biological functions including aspects of both aerobic and anaerobic metabolism (Alberts et al. 1994; Halliwell and Gutteridge 1999). The ratio between the two metabolites is a potential indicator of metabolic state and intracellular redox potential (Lin and Guarente 2003). Therefore NAD^+ concentrations are likely to be relevant during cell viability loss and to contribute to K-value calculations in the non-viable tissue.

Measurements of these metabolites is achieved by HPLC based assays that, although useful, are time consuming and expensive and therefore used primarily for research purposes (Ryder 1985; Furst and Hallstrom 1992).

1.5 Free radical production and oxidative damage in skeletal fish muscle

Aerobic metabolism, despite its efficiency, is known to produce highly reactive free radicals predominantly through leakage of electrons from the electron transport chain and the subsequent reduction of molecular oxygen to form superoxide ($\text{O}_2^{\bullet-}$) and H_2O_2 (Nohl and Hegner 1978; Cadenas and Davies 2000; Kudin et al. 2005). H_2O_2 can then be cleaved by transition metals through Fenton chemistry to produce the highly damaging hydroxyl radical ($\bullet\text{OH}$) (Halliwell and Gutteridge 1999; Sachdev and Davies 2008). Although a number of processes contribute to the total cellular pool of superoxide and H_2O_2 , during normal metabolic processes mitochondrial generation is quantitatively the biggest contributor. It is generally thought that in the resting mitochondria (state 4), between 1 and 4% of total oxygen consumption will result in the generation of superoxide (Jackson 1994;

Cadenas and Davies 2000; Sachdev and Davies 2008). As such the greatly increased flux of electrons through the mitochondria during exercise could lead to increased superoxide production. However, the transition of the mitochondria to the active respiratory state (state 3) during exercise reduces superoxide production considerably, and in the anoxic state 5 there is no superoxide production (Cadenas and Davies 2000).

A secondary source of free radicals is xanthine oxidase. This is a modified form of xanthine dehydrogenase produced during periods of ischemia and/or hypoxia. It metabolizes hypoxanthine generated by the breakdown of adenosine monophosphate (AMP) to xanthine and eventually uric acid. This process, unlike that used by xanthine dehydrogenase, utilizes oxygen as an electron acceptor, generating xanthine and superoxide (Chambers et al. 1985; McCord 1988; Jackson 1994; Sachdev and Davies 2008). As oxygen is required for this pathway, it is most pertinent when oxygen is available to the tissue such as during reperfusion or storage in an oxygen rich environment.

A loss of calcium homeostasis can lead to the release of free fatty acids and disruption of membranes through the activation of phospholipases and proteolytic enzymes (Jackson 1994). The release of iron from haemoglobin and myoglobin allows it to catalyze the Haber-Weiss and Fenton reactions and is a known source of free radicals (Halliwell and Gutteridge 1999). This type of tissue damage has been shown to take place following spinal cord impact injuries (Liu et al. 2003) and is likely to occur following the damage of any iron rich tissue. Iron catalyzed oxidation has also been shown to be an important factor in fish and other meat products (Richards et al. 2002; Tokur and Korkmaz 2007).

Nitric oxide (NO) has been shown to play an important role in the regulation of vascular tone in mammals (Torreilles 2001). It also influences proteolytic enzyme activity and is involved in calcium homeostasis (Michetti et al. 1995; Hare 2003; Warner et al. 2005). NO is a free radical compound and reacts extremely rapidly with superoxide to form ONOO⁻, which can subsequently be protonated to form HOONO and then dissociated to NO₂ and [•]OH (Khanna et al. 2005). Therefore its production *in vivo* can contribute to the redox environment. It has been shown to influence the tenderness of certain types of meat (Warner et al. 2005) and play a role in reperfusion injury in skeletal muscle (Khanna et al. 2005). Although the role of NO in fish is more uncertain (Eddy 2005), NO donors have been shown to vasodilate, and NO inhibitors vasoconstrict rainbow trout coronary systems (Mustafa et al. 1997). This indicates that NO does play a role in regulating vascular tone in salmonids and may therefore also be involved in oxidative processes in fish tissues.

Although during normal metabolic processes mitochondrial superoxide generation is the dominant process for free radical production, the relative contributions of each of these free radical generating pathways will be dependent on a number of factors. In meat products in particular the onset of ischemia during harvesting and the subsequent processes leading to cell death are likely to cause factors that are not normally associated with oxidative processes in viable tissues to become major contributors in the non-viable tissue (Morrissey et al. 1998).

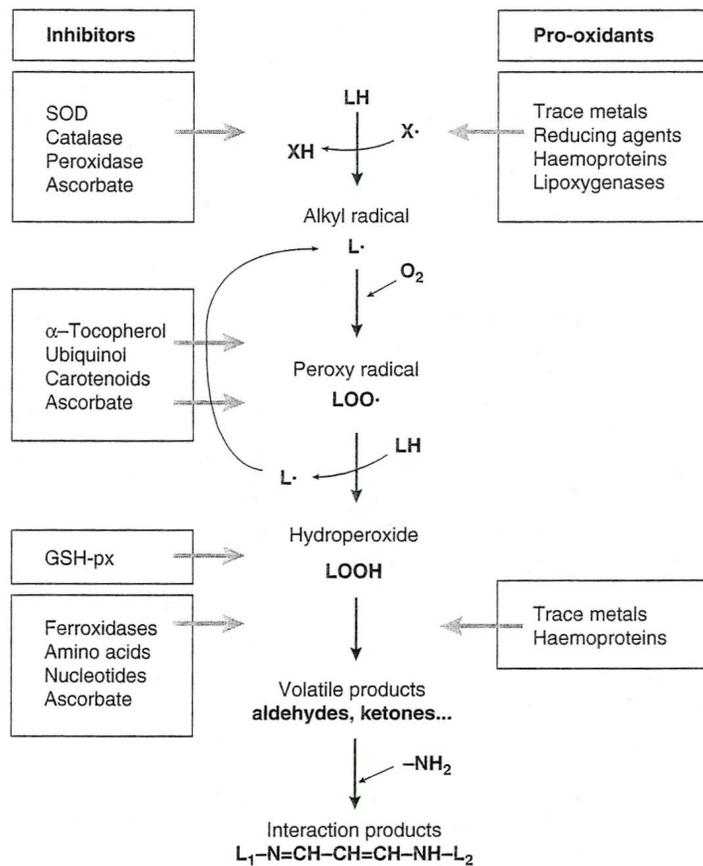


Figure 1.4 Schematic illustration of the lipid autoxidation process including some of the native inhibitors and pro-oxidants found in fish tissues. LH = fatty acid, X· = initiator, L· = alkyl radical, LOO· = peroxy radical, LOOH = hydroperoxide, SOD = superoxide dismutase, GSH-px= glutathione peroxidase. (from Undeland 2001)

When considering likely sources of free radical production the properties of the tissue in question must also be established. In the case of teleost red muscle the tissue is packed with mitochondria and contains large quantities of intracellular myoglobin and lipid (Kiessling et al. 2006). This provides for a large source of mitochondrially generated free radicals if oxygen is present in the viable tissue. As viability is lost the release of iron

from the intracellular myoglobin and any haemoglobin from blood residues are also able to contribute to free radical production. The large quantities of unsaturated intracellular lipids are particularly susceptible to oxidation. The combination of these factors makes teleost red muscle tissue particularly susceptible to oxidation reactions that produce unfavourable characteristics in the tissue (Undeland 2001). Comparatively, the white muscle has relatively few mitochondria and smaller quantities of lipid (Kiessling et al. 2006) and very small quantities of myoglobin (Richards and Hultin 2002), making it generally more resistant to these processes. However, more rapid production of tertiary lipid oxidation products has been shown in teleost white muscle than in teleost red muscle (Undeland 2001). The relative susceptibilities of different fish tissue types to oxidation have been shown in herring fillets (Undeland et al. 1998; Undeland 2001).

Free radicals that are not neutralized by endogenous antioxidant systems can damage important cellular components. The primary products of protein and lipid oxidation are protein and lipid hydroperoxides (ROOH). These are odourless and tasteless compounds and are relatively short lived, being rapidly oxidized to secondary products (Undeland 2001). The secondary products of lipid oxidation include volatile aldehydes and ketones that are responsible for some of the fishy, oily and rancid odours and flavours found in stored fish (Undeland 2001). The further oxidation of protein hydroperoxides produces carbonylated proteins, which are associated with impaired protein function and increased protein carbonyl concentrations are found in a number of disease pathologies (Shacter 2000). Carbonylated proteins have also been shown to increase during the storage of fish fillets (Kjaersgard et al. 2006).

Detection of free radical damage is predominantly achieved by measuring the products of oxidative processes. Protein and lipid hydroperoxides can be reduced with ferrous ions, and the resulting ferric ions chelated with xylenol orange to form a complex detectable by spectroscopy at 560 nm (Gay et al. 1999; Gay and Gebicki 2002; Gay and Gebicki 2003). Protein carbonyls can be detected by derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Quinlan et al. 1994; Shacter 2000) and malondialdehyde (MDA), a secondary product of lipid oxidation, can be detected by derivatization with thiobarbituric acid (TBA) (Wong et al. 1987; Agarwal and Chase 2002). These are some of the most general and widely used techniques. However, due to the non-specific nature, the wide range of products produced through the oxidative modification of proteins and lipids and the relative instability of some of these compounds, these measurements are only partially indicative of oxidative damage. Attempts have been made to create an assay that measures

the total antioxidant potential of a tissue homogenate (Arnao et al. 2001), however the generation of antioxidant compounds such as uric acid during the degradation of fish products mean that the results produced by these techniques would be very difficult to interpret and of limited use. Unfortunately there is no single technique that allows the accurate measurement of all of the products of oxidative processes in biological tissues and therefore a combination of techniques is often utilized (Halliwell and Gutteridge 1999).

1.6 Endogenous antioxidants in skeletal fish muscle

The general definition now used to define the term 'antioxidant' is 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell et al. 1995; Halliwell and Gutteridge 1999). *In vivo* and in food products made up of animal tissues the substrate comprises almost all components of the tissue. Endogenous antioxidants include antioxidant compounds and systems that occur naturally, either through endogenous manufacture and regulation and/or dietary sources, and are important for normal metabolic function in living tissues. The metabolic function and potential contribution of endogenous antioxidants to the oxidative stability of seafood products is discussed below.

1.6.1 Antioxidant enzymes

The production of free radicals through normal metabolic processes requires cells to have efficient and adaptable endogenous antioxidant systems to maintain homeostasis. Generally there is a very good match between the antioxidant capacity of a tissue or animal and its environment, with red muscle fibres showing higher activities of these antioxidant enzymes than white muscle fibres (Ji 2008). A number of cellular antioxidant defenses consist of antioxidant enzymes. The superoxide dismutases (SODs) are metal-containing enzymes that catalyze the conversion of superoxide to H_2O_2 and O_2 (Halliwell and Gutteridge 1999). CuZnSOD is found predominantly in the cytosol of most eukaryotic cells, with smaller quantities in the nucleus, lysosomes and between the inner and outer mitochondrial membranes. MnSOD is almost entirely located in the mitochondria (Halliwell and Gutteridge 1999). The relative activities and locations of SODs are species and tissue specific. Over expression of CuZnSOD in *Drosophila* and mice increases resistance to oxygen toxicity (Huang et al. 1992; Le Bourg 2001), while mice lacking

MnSOD suffer acute and fatal pathologies consistent with major mitochondrial damage (Williams et al. 1998; Huang et al. 2001). This indicates their importance, particularly of MnSOD, in maintaining redox homeostasis and mitochondrial function.

Catalase is a haem-containing enzyme that catalyzes the conversion of H_2O_2 to H_2O and O_2 . Aerobic cells generally contain some catalase activity with liver, and to a lesser extent erythrocytes, showing high activities (Halliwell and Gutteridge 1999). As H_2O_2 is relatively stable and diffuses easily, erythrocytes can help to protect other tissues. The catalase activity in animal cells is mainly found in peroxisomes, which contain a number of H_2O_2 generating enzymes such as those involved in the β -oxidation of fatty acids. Mitochondria do not contain significant amounts of catalase, meaning it is not involved in the removal of H_2O_2 in the mitochondria (Halliwell and Gutteridge 1999). Mutations resulting in catalase deficiency do cause obvious disease pathologies (Goth 2001) and over expression provides extra protection against specific stressors (Xu et al. 1999). Therefore catalase is an important component of, but not vital for aerobic metabolism.

Glutathione peroxidase (GSH-px) is a selenium containing enzyme that catalyzes the reaction of H_2O_2 and reduced glutathione (GSH) to form H_2O and glutathione disulphide (GSSG). GSSG is then reduced back to GSH through the oxidation of NADPH (Halliwell and Gutteridge 1999). GSH-px is found in many animal tissues and is also able to act on fatty acid peroxides, reducing them to an alcohol (Halliwell and Gutteridge 1999). GSH-px activity in coho salmon can be increased through selenium supplementation (Felton et al. 1996) and is the only antioxidant enzyme whose activity can be influenced by diet.

Environmental factors however, can contribute to the relative activities of antioxidant enzymes. Up regulation of the antioxidant enzymes has been shown in response to exercise, hypoxia and temperature stress (Selman et al. 2000; Lushchak et al. 2005; Pinho et al. 2006; Gomez-Cabrera et al. 2007; Lambertucci et al. 2007). In rainbow trout GSH-px and catalase activities in the blood were increased by temperature, handling and low water stress (Ozmen et al. 2007). This indicates the importance and flexibility of these endogenous systems in maintaining homeostasis in response to changing environmental factors.

The role these enzymes play during the storage of meat products is less clear. There are a number of technical difficulties associated with applying antioxidant enzymes in food technology and therefore they have no current practical applications (Meyer and Isaksen 1995). Changes in the activities of these enzymes have been documented during

the storage of meat products (Hernandez et al. 2002; Hoac et al. 2006) and it is likely they play a role in maintaining the oxidative stability of the meat as long as their activity is retained. The relevance of these observations to a tissue with a low aerobic capacity such as teleost white muscle is uncertain. However, reduced GSH-px activity has been shown to correlate with the onset of lipid oxidation in herring fillets (Undeland et al. 1999).

1.6.2 Glutathione

GSH is involved in a number of important processes distinct from its action as a cofactor for GSH-px. These include acting as a cofactor in other enzymatic pathways, ascorbic acid metabolism, preventing oxidation and subsequent cross linking of protein thiol groups, repair of oxidized sites, protein folding and degradation of proteins containing disulphide bonds (Halliwell and Gutteridge 1999). It has also been shown to react directly with a number of free radicals and, given its relatively high intracellular concentrations, these reactions are likely to occur *in vivo*. GSH can be manufactured enzymatically from cysteine and is in a constant state of turnover due to its relatively short half life. Deficient organisms show general sensitivity to toxins and radiation but associated pathologies are not fatal (Halliwell and Gutteridge 1999). GSH concentrations in fish have been shown to change in response to temperature (Leggatt et al. 2007) and administration of GSH directly has been shown to alter the status of some antioxidant defenses (Otto et al. 1997) indicating that GSH metabolism is flexible. Given its radical scavenging ability, GSH present in meat products is likely to contribute to oxidative stability.

1.6.3 Vitamin C

Vitamin C (ascorbate) is synthesized in plants and in the liver of a number of animals from glucose using the enzyme gulonolactone oxidase. This reaction also results in production of H₂O₂ meaning that, although ascorbate functions as an antioxidant, high levels of production could result in oxidative stress (Halliwell and Gutteridge 1999). Plant based food sources contribute significantly to many animals' *in vivo* vitamin C pools and is the only source for animals that can not manufacture it. Ascorbate is required as a cofactor for a number of enzymes, two of which are involved in collagen synthesis meaning deficiency results in structurally weakened tissues and blood vessels (Halliwell and Gutteridge 1999). The ability of ascorbate to act as a reducing agent is well documented and it has been shown to act as an efficient antioxidant in meat products (Formanek et al. 2003; Mielnik et

al. 2003; Mitsumoto et al. 2005; Banon et al. 2007). However, the ability of ascorbate to reduce transition metals means that under some circumstances, particularly at low concentrations, it can have a pro-oxidant action (Buettner and Jurkiewicz 1996). This is also a property of other reducing agents such as GSH.

The donation of an electron from ascorbate produces an ascorbyl radical that is relatively stable, which provides the basis for its antioxidant properties (Halliwell and Gutteridge 1999). This can be further oxidized to form dehydroascorbate (DHA). In a number of cells including skeletal muscle, both ascorbate and DHA are actively transported into the cell where DHA can be enzymatically recycled back to ascorbate with the subsequent loss of GSH (Savini et al. 2005). The ability of extra dietary ascorbate to elevate vitamin E concentrations in the liver of yellow perch (Lee and Dabrowski 2003) indicates that it not only acts as an antioxidant *in vivo*, but also that it interacts with and recycles other antioxidants as shown by Buettner (1993).

Adequate dietary intake of ascorbate also improves growth rates of aquacultured fish (Li et al. 2007), but its relatively low concentrations in teleost white muscle tissue mean that it may not play a significant role in the quality of fillets (Espe et al. 2002; Ruff et al. 2003). Morrissey et al. (1998) commented that ascorbate supplementation appears to have little effect on meat stability. Prolonged administration of ascorbate and vitamin E in the diet has been shown to improve the oxidative stability of both beef and fish (Schaefer et al. 1995; Ruff et al. 2003), but this may be primarily related to the elevated vitamin E concentrations in the respective tissues.

1.6.4 Vitamin E

Vitamin E (RRR- α -tocopherol) is the most biologically effective form of a family of eight tocopherols, which are lipid soluble substances that inhibit lipid peroxidation. The hydrophobic tail anchors the molecules into the mitochondrial and cellular membranes leaving the -OH group responsible for its antioxidant action at the membrane interface (Halliwell and Gutteridge 1999). Tocopherols inhibit lipid peroxidation through the rapid scavenging of lipid peroxy radicals (LO_2^*) generating a tocopherol radical in the process, which can then be recycled back to its reduced state by ascorbate (Buettner 1993). The role of vitamin E *in vivo* appears to be predominantly as an antioxidant. There are few symptoms of deficiency other than a general susceptibility to oxygen toxicity and evidence of increased lipid peroxidation in some tissues. However, this may be due to the prolonged

period of dietary deficiency required to reduce tissue vitamin E concentrations, particularly in the brain, where chronic deficiency eventually results in neurological damage (Halliwell and Gutteridge 1999). More recently the nature of this role *in vivo* has been questioned by work showing that vitamin E has other important cellular functions (Azzi et al. 2004; Zingg and Azzi 2004). Other researchers maintain that these are mediated via its antioxidant function and comments by Brigelius-Flohe and Davies (2007) point out that there is much to be done to advance understanding of the role of vitamin E *in vivo*.

The inclusion of vitamin E in the feed of farmed animals has been shown to reduce signs of lipid peroxidation in tissues such as the liver and also to improve the oxidative stability of the post-mortem tissue (Schaefer et al. 1995; Morrissey et al. 1998; Lee and Dabrowski 2003; Ruff et al. 2003; Huang et al. 2004). In trout fillets tissue vitamin E concentrations have been shown to affect the oxidative stability of the tissue during storage (Jensen et al. 1998). This is likely to also be of relevance to salmon fillets during storage.

1.6.5 Carotenoids

Carotenoids are a family of lipid soluble compounds that serve as precursors to vitamin A, which is essential for cell growth and differentiation (Halliwell and Gutteridge 1999). They are characterized by the red yellow and orange hues that are common in nature including many sea foods (Shahidi et al. 1998). These colours play a major role in determining the acceptability of food products (Shahidi et al. 1998). Many play key antioxidant roles in plants and β -carotene has been shown to protect model systems from lipid peroxidation *in vitro* at low O₂ concentrations. Generally, carotenoids are good quenchers of singlet oxygen (¹O₂), however the relevance of these reactions *in vivo* is still somewhat uncertain (Halliwell and Gutteridge 1999). Recent work indicates β -carotene from an algal source was able to confer protection against oxidative stress and that this included supporting the activities of endogenous antioxidant enzymes (Murthy et al. 2005).

In salmon, the carotenoid astaxanthin is primarily responsible for the appealing pink colour of the flesh. Although it has not been shown to impact directly on the taste/texture aspects of fresh salmon fillets, sensory panels show a preference for pigmented flesh (Sigurgisladdottir et al. 1994). The relationship between tissue astaxanthin concentrations and lipid peroxidation in salmonid tissue during storage appears to be dependent on species and storage conditions. However, there is evidence that astaxanthin contributes to the oxidative stability of the lipid fraction of salmonid tissues during storage

(Andersen et al. 1990; Bjerkeng and Johnsen 1995; Jensen et al. 1998; Aubourg et al. 2005).

1.6.6 Coenzyme Q

Coenzyme Q (ubiquinol) is predominantly involved in the mitochondrial electron transport chain. It aids in the prevention of lipid oxidation in the inner mitochondrial membrane, ferries electrons through the electron transport chain and is a primary source of intracellular superoxide (Sohal et al. 2006). It is also a component of cell membranes and lipoproteins, but its function is not well defined in these locations. Coenzyme Q can inhibit lipid peroxidation through the scavenging of peroxy radicals and regenerate α -tocopherol from its radical form, which is likely to be relevant to its function *in vivo* (Stoyanovsky et al. 1995; Halliwell and Gutteridge 1999; James et al. 2004). Due to its important role in aerobic metabolism it has been suggested that measurements of coenzyme Q concentrations are indicative of mitochondrial function and redox status (Miles et al. 2005a; Miles et al. 2005b). Coenzyme Q deficient yeast are sensitive to damage by oxidized PUFA products (Poon et al. 1997) and deficient mice die as embryos (Nakai et al. 2001) indicating its important role in aerobic metabolism.

Both forms of coenzyme Q (Q9 and Q10) are found in rainbow trout with coenzyme Q10 being the predominant form (Mattila and Kumpulainen 2001). The role it plays during the storage of fish fillets is somewhat uncertain. However, its radical scavenging ability and interaction with α -tocopherol mean that it is likely to play a role in protecting the lipid fraction of the tissue from oxidation during storage.

1.6.7 Histidine containing dipeptides

Many tissues including the skeletal muscle of teleost fishes contain histidine containing amino acid dipeptides such as carnosine and anserine. Anserine in particular has been shown to be a component of salmonid white muscle (Abe 1983; Suzuki et al. 1990; Chan and Decker 1994). These compounds are weak inhibitors of lipid oxidation *in vitro* but their role *in vivo* is uncertain. They do not exhibit the pro-oxidant activity of histidine and may allow a safe means of accumulating histidine, while acting as an intracellular pH buffer (Halliwell and Gutteridge 1999). Dietary supplementation was unsuccessful at elevating tissue carnosine concentrations in mice (Chan et al. 1994), but post-mortem

carnosine addition has been suggested as a means of improving the lipid stability of meat products (Morrissey et al. 1998).

1.7 Exogenous antioxidants

There is some overlap between what can be defined as an exogenous antioxidant and an endogenous antioxidant. For our purposes an exogenous antioxidant is an antioxidant that is not important for normal metabolic function in animal tissues, or in circumstances relating to the processing and storage of meat and seafood products, an antioxidant that is introduced into the tissue during harvesting or processing to prevent or slow the oxidative degradation of that tissue. An obvious example of a compound that can be described as both an endogenous and exogenous antioxidant is ascorbate, which is an essential component of normal metabolic function and can also be used as an exogenous antioxidant for the stabilization of processed meat products (Mielnik et al. 2003; Banon et al. 2007).

1.7.1 Phenols

Phenolic compounds contain one or more benzene rings with either one or two attached –OH groups (Halliwell and Gutteridge 1999). Many phenols have a strong antioxidant action *in vitro* and inhibit lipid peroxidation via peroxy radical scavenging (Davcheva et al. 1995; Cuvelier et al. 2003). Some phenols contain transition metal chelating structures that prevent or reduce the ability of the bound transition metal to contribute to redox reactions (Halliwell and Gutteridge 1999). Phenols are found in many plants and can function as precursors in biosynthetic pathways or absorb ultraviolet radiation. They often contribute greatly to the colour and taste of plant material such as the rich red/blue of the anthocyanins found in berries and the well known flavouring compound vanillin. Herbs and spices containing phenols have been used to preserve foods for much of recorded human history (Lindberg Madsen and Bertelsen 1995). Many phenol rich plant extracts have been shown to inhibit lipid peroxidation and have shown potential in preserving and prolonging the shelf life of fish and meat products (Tozer 2001; Banon et al. 2007; Raghavan and Richards 2007). Phenols have also been shown to have vasoactive properties (Packer et al. 1999; Deliorman et al. 2000; Dell'Agli et al. 2004; Ghayur et al. 2005) and to interact with endogenous antioxidants (Kadoma et al. 2006) and therefore

may be useful in preserving tissue function in perfusion preparations via their antioxidant function and their potential to improve vascular tone.

The phenolic compound isoeugenol (the active ingredient in the aquatic anaesthetic AQUI-S™) is now used in the aquaculture industry as an anaesthetic to reduce stress and exhaustion in the fish during normal animal husbandry and harvesting procedures (Wagner et al. 2002; Iversen et al. 2003; Bosworth et al. 2007). Isoeugenol has been shown to enter the bloodstream and tissues of anaesthetized fish (Kildea et al. 2004; Meinertz et al. 2006) and to have vasoactive and antioxidant properties (Davcheva et al. 1995; Uchida et al. 1996; Guha and Priyadarsini 2000; Ito et al. 2005; Rothwell and Forster 2005).

Although isoeugenol has been shown to be cytotoxic at concentrations between 100 and 300 μM in human cell culture studies (Burkey et al. 2000; Atsumi et al. 2005), it is rapidly removed from fish tissues following exposure (Kildea et al. 2004; Meinertz et al. 2006). Isoeugenol has a half life in rats following intravenous administration of approximately 12 minutes (Badger et al. 2002). It can also be consumed by rats in quantities up to 250 mg/Kg/day with no adverse effects (George et al. 2001; Badger et al. 2002).

The antioxidant property of isoeugenol, like other phenolic compounds, is mediated by an –OH group on the benzene ring. The benzene ring contains a series of conjugated double bonds, which in the case of isoeugenol extend onto the propenyl side chain. This allows electron delocalization through the conjugated double bonds increasing the stability of the phenoxyl radical (Rajakumar and Rao 1993; Brandwilliams et al. 1995; Davcheva et al. 1995). Isoeugenol has also shown antioxidant activity in transition metal induced oxidation systems (Rajakumar and Rao 1993; Uchida et al. 1996; Ito et al. 2005). Isoeugenol has both metal reducing and metal chelating properties making the exact mechanism of its action difficult to determine *in vivo* (Ito et al. 2005; Nenadis et al. 2007).

Isoeugenol may have a number of potentially important effects in harvested fish tissues due to the rested metabolic state of the tissue and the potential for antioxidant function within the tissue. These will be addressed in harvested tissues from chinook salmon in subsequent chapters of this thesis.

1.7.2 Synthetic antioxidants

The chemical structures that provide for antioxidant action can be utilized to formulate synthetic antioxidants that can act as radical scavengers and/or transition metal chelators

(Halliwell and Gutteridge 1999). Examples of synthetic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trolox and propyl gallate. BHA and BHT have been added to foodstuffs to inhibit lipid oxidation and are successful in doing so (Verhagen et al. 1991; Lindberg Madsen and Bertelsen 1995). However, high doses of BHA have been implicated in the formation of forestomach cancer in rats, but there is no evidence that doses received by humans in foodstuffs have medical implications (Botterweck et al. 2000). However, these suggestions mean that the addition of synthetic antioxidants to foodstuffs is beginning to be unacceptable to consumers and much recent work has focused on replacing them with naturally derived compounds or extracts (McArthy et al. 2001; Raghavan and Richards 2007). The addition of synthetic antioxidants during processing has proved successful for inhibiting lipid oxidation in meat (McArthy et al. 2001; Mielnik et al. 2003). Dietary intake of synthetic antioxidants by fish however, did not improve the oxidative stability of the tissue (Gatlin et al. 1992). This was likely due to the antioxidants being rapidly metabolized and excreted. Although it is likely that synthetic antioxidants added post harvest would contribute to the oxidative stability of seafood products, the acceptability of such products to consumers is questionable.

1.8 Research aims for this thesis

As discussed above, this thesis focuses on aspects of metabolic function in teleost skeletal muscle and its relationship to oxidative processes and antioxidant status. In particular we aim to further develop the chinook salmon tail perfusion model developed by Janssen (2003) and look for ways to improve oxygen delivery to the tissue with the ultimate aim of inducing and maintaining a hypometabolic state. This goal is pertinent to a number of practical applications including preserving animal tissue based foodstuffs and the storage of tissues for transplantation. Of particular importance to this goal is to examine the oxidative processes occurring in the red and white skeletal muscle tissues during the post-harvest period in which cell death occurs and determine if oxidative damage and/or the loss of key antioxidants such as vitamins C and E plays a role. As harvested tissue is ischemic and deprived of oxygen there is also the potential that these processes may occur at a later stage during storage and be more related to the degradative processes that occur in animal tissues that lead to spoilage and rancidity. The dominant metabolic pathway (i.e. aerobic versus anaerobic) of a specific tissue may play a determining role.

We also wish to examine the effect of antioxidants in controlling oxidation in the perfusion model and stabilizing and prolonging the shelf life of harvested tissue. Of particular interest are both the physiological and biochemical effects on post-harvest metabolism and long term storage properties in tissues harvested from fish in a rested state using the aquatic anaesthetic AQUI-S™. As the active ingredient of AQUI-S™ is a lipid soluble compound (isoeugenol) with antioxidant properties, the resistance of tissues harvested from rested fish to oxidation will also be of interest.

2 - Materials and Methods

This chapter describes all the equipment, suppliers and methodology used throughout this work. More specific information on methods is contained within subsequent chapters. Also included are descriptions of method development and evaluations of their suitability for use in this work.

2.1 Experimental animals

Female chinook salmon (*Oncorhynchus tsawytscha*) were supplied by Isaac Salmon Farm (McLeans Island, Christchurch, New Zealand) and were grown to harvest weight on a diet of fortified fish meal in outdoor concrete raceways. A continuous flow of freshwater at a temperature of approximately 12°C is supplied from a bore hole in the Canterbury plains. Because ground water is used there is little seasonal temperature variation.

2.1.1 Exhausted harvesting

Fish were sampled directly from the farm's harvesting procedures. Generally, fish were separated in the raceway using a fine mesh divider. This was moved along the raceway until the fish were crowded into one end. The divider was locked in place, a large net was lowered into the water and a number of fish were removed. The fish were then placed in a large crate filled with water and gassed with CO₂ until they ceased struggling. They were then removed one by one and were killed and bled. For our purposes fish were removed from the net and not subjected to CO₂ anaesthesia. They were killed by cranial impact and transported back to the University of Canterbury on ice for fillet preparation.

2.1.2 Rested harvesting

A small number of fish were transferred from the open raceways to an indoor holding facility on the premises. They were placed in circular tanks covered with shade cloth with a diameter of 1.9 m and a water depth of approximately 630 mm. Natural lighting and a flow of clean fresh bore water was maintained to the tanks at all times. The fish were kept under these conditions for 5-7 days before being used for experimental work.

To achieve a rested harvest, the freshwater feed to the holding tank was shut off and the water was kept circulating and aerated by standard aquarium water and aeration pumps. A stock solution of 25 ml of AQUI-S™ (AQUI-S New Zealand Ltd., Lower Hutt) was made up in approximately 7 litres of water. This was placed in a bucket with a slow gravity fed drain into the holding tank. This brought the concentration of AQUI-S™ up to approximately 15 ppm over 20 minutes. This allowed the slow onset of anaesthesia and a minimization of the discomfort and stress associated with sudden exposures to high concentrations of AQUI-S™. After monitoring the behaviour of the fish, the concentration of the AQUI-S™ in the tank was raised to 17-19 ppm over 5 minutes using a further 5 ml of AQUI-S™. Generally, the fish had lost equilibrium, were no longer swimming, ventilation rates were approximately $\frac{1}{3}$ of normal resting rates and were unresponsive to handling. If any responses to handling were noted that individual was not used for experimental work. Fish were either transported live in sealed plastic bags on ice containing approximately 10 litres of water with 17 ppm AQUI-S™ and 10 litres of medical grade O₂, or were killed and transported on ice to the University of Canterbury.

2.2 Perfused whole tails

NaCl	8	L-glutamic acid	0.051
KCl	0.157	L-glutamine	0.059
CaCl ₂ (2H ₂ O)	0.171	L-aspartic acid	0.003
MgCl ₂ (6H ₂ O)	0.204	DL-carnitine	0.01
NaHCO ₃	1.092	BSA (Fraction V)	20
BES	0.213	PVP (MW 40 000)	30
Glucose	1.802		

Table 2.1 Contents and masses for freshwater teleost saline (modified Rees-Simpson saline). Saline was made up in distilled water, adjusted to pH 7.4 with 10 M NaOH and filtered through a double layer of filter paper. All values expressed as g L⁻¹.

Live rested anaesthetized fish were transported to the University of Canterbury. They were then pithed (brain and spinal cord ablation) with a traditional Japanese ike-jimi tool and 1 ml of heparinized saline was injected into the dorsal aorta through the roof of the mouth. Perfusions were carried out using the technique of Janssen (2003) (M.Sc. thesis, University of Canterbury, Christchurch). Two lateral incisions were made down through the dorsal surface of the fish, one through the centre of the dorsal fin and the second immediately posterior to the dorsal fin until contact with the spinal column was made. These incisions

were continued around the entire circumference of the animal leaving the spinal column intact. The soft tissue between the two incisions was completely trimmed away leaving the spinal column exposed. The spinal column was then severed at the anterior end of the exposed section. The tail preparation was weighed and placed in a temperature controlled bin at $15 \pm 0.5^\circ\text{C}$. 1.5-3 mm diameter silicon cannulas (Microtube Extrusions, North Rocks, NSW), size depending, were inserted into the dorsal aorta and the caudal vein and were sealed with two firm ties around the exposed spinal column. Freshwater teleost saline, made up as shown in table 2.1 and continuously bubbled with medical grade oxygen, was pumped into the tail preparation through the dorsal aorta using a Gilson Minipuls 3 peristaltic pump (Gilson Inc., Middleton, WI) at a rate of 2.5 ml/min/100g tail weight (shown in Figure 2.2). A pressure transducer connected via a t-junction to the flow line continuously monitored and recorded perfusion backpressure through a Powerlab 4SP data recorder (ADInstruments Pty Ltd., Bella Vista, NSW). Tissue sampling and pH

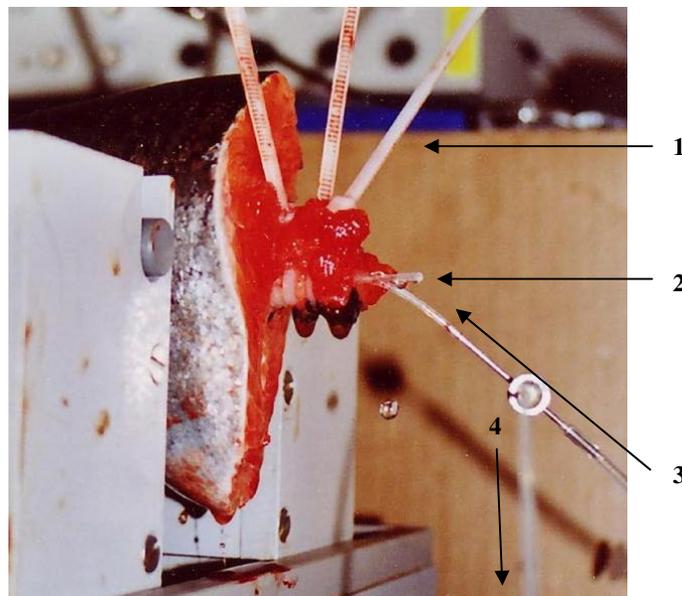


Figure 2.2 Photograph of a cannulated and perfused salmon tail. 1 Cable ties used to seal blood vessels around the inserted cannulas. 2 Cannulated caudal vein. 3 Flow of saline from the peristaltic pump into the cannulated dorsal aorta. 4 Static fluid filled line connected to the pressure transducer.

measurements were carried out as specified in Janssen (2003). Two 15 mm thick transverse slices of the trunk were cut from the posterior end of the remaining head section. The second of these slices was taken and its cut surface pH was measured using a Radiometer PHM 84 (Radiometer Pacific Ltd., Takapuna, Auckland) equipped with a Sensorex 450C pH probe (Sensorex, Garden Grove, CA) by taking the mean of six

concurrent measurements. Samples from the lateral band of red muscle and the white muscle approximately 15 mm lateral to the spinal column (D block) were extracted, immediately freeze clamped and stored at -80°C . Upon completion of the perfusion the tail was removed from the temperature controlled bin, weighed and the sampling procedure repeated by taking two 15 mm slices from the anterior end of the tail preparation. The unexposed slice was used for sampling.

2.3 Tail fillets stored in air

2.3.1 Oxidation products and loss of tissue antioxidants

Rested, exhausted and exhausted salmon that had subsequently been put through the rested harvesting procedures were killed by a blow to the head and transported back to the University of Canterbury on ice. Fillets were cut running from the dorsal fin to the tail and cut surface pHs were taken using a Radiometer PHM 84 equipped with a Sensorex 450C pH probe as a mean of six concurrent measurements, 3 each side of the spine from the anterior end of the fillet spaced at approximately 10 mm intervals. A sample was then cut from the fillet and freeze clamped. The fillets were placed in a temperature controlled bin at $15 \pm 0.5^{\circ}\text{C}$. pH measurements and surface tissue samples from the D blocks of tissue at undisturbed locations were taken after 6, 12, 24, 30, 36, 48, 54, 60, 72 and 96 hours storage. The muscle from the posterior third of the fillet was not sampled. Samples were stored at -80°C for analysis.

2.3.2 Creatine compounds, nucleosides, nucleotides and related bases

Rested and exhausted salmon were treated as described above with measurements taken at the 0, 3, 6, 12, 24, 30 and 36 hour time points. Two samples were taken at 30 hours, one from the surface of the fillet and one immediately below from the unexposed centre of the fillet.

2.4 Biochemical methodology

2.4.1 Reagents

All reagents were of analytical grade or better and unless stated otherwise were made up in deionized and ultrafiltered water from a Milli-Q filtration system. All HPLC mobile phases containing salts were vacuum filtered through 0.45 μm filter membranes and degassed by sonication.

α -tocopherol	Sigma Chemical Co; St Louis, MO, USA
β -Nicotinamide adenine dinucleotide (NAD ⁺)	Sigma Chemical Co.
1,1,3,3-tetramethoxypropane (malonaldehyde)	Sigma Chemical Co.
2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)	Arcos; NJ, USA
2,4-dinitrophenyl hydrazine (DNPH)	Sigma Chemical Co.
2-mercaptoethanol	Sigma Chemical Co.
2-thiobarbituric acid (TBA)	Sigma Chemical Co.
Acetic acid	J.T. Baker; NJ, USA
Acetonitrile	BDH Chemicals Ltd; Palmerston North, NZ
Adenosine diphosphate (ADP)	Merck Ltd; Poole, England
Adenosine monophosphate (AMP)	Sigma Chemical Co.
Adenosine triphosphate (ATP)	Sigma Chemical Co.
Ascorbic acid	Sigma Chemical Co.
AQUI-S™	BDH Chemicals Ltd.
Bovine serum albumin (BSA)	AQUI-S NZ Ltd; Lower Hutt, NZ
Butylated Hydroxytoluene (BHT)	Invitrogen; Auckland, NZ
Calcium chloride	Sigma Chemical Co.
Chloroform	BDH Chemicals Ltd.
Creatine	Asia Pacific Chemicals Ltd; Auckland, NZ
Creatine Phosphate	Merck Ltd.
Creatinine	Sigma Chemical Co.
D-glucose	Sigma Chemical Co.
DL-carnitine	Biolab Ltd; Vic, Australia
Dodecyltrimethylammonium chloride	Sigma Chemical Co.
Ethanol	Sigma Chemical Co.
Ethyl acetate	BDH Chemicals Ltd.
Ethylendiaminetetraacetic acid (EDTA)	BDH Chemicals Ltd.
Glutathione (GSH)	BDH Chemicals Ltd.
Guanidine Hydrochloride	Sigma Chemical Co.
Heparin	Sigma Chemical Co.
Hydrochloric acid (HCl)	Merck Ltd.
Hypoxanthine	Sigma Chemical Co.
Inosine	Sigma Chemical Co.
Inosine monophosphate (IMP)	Sigma Chemical Co.
L-aspartic acid	Sigma Chemical Co.
L-glutamic acid	Sigma Chemical Co.
L-glutamine	Sigma Chemical Co.
Magnesium chloride	BDH Chemicals Ltd.
Methanol	BDH Chemical Ltd.
Monobromobimane (MBB)	Merck Ltd.
	Sharlau Chemie SA; Gato Perez, Spain
	Sigma Chemical Co.

N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)	Sigma Chemical Co.
n-hexane	Asia Pacific Chemicals Ltd.
Nitrogen Gas	BOC Gasses
O-phthalaldehyde (OPA)	Sigma Chemical Co.
Orthophosphoric acid (85%)	BDH Chemicals Ltd.
Perchloric acid (PCA)	BDH Chemicals Ltd.
Polyvinylpyrrolidone MW 40 000 (PVP)	Sigma Chemical Co.
Potassium carbonate	Merck Ltd.
Potassium chloride	Merck Ltd.
Sodium acetate	Merck Ltd.
Sodium chloride	BDH Chemicals Ltd.
Sodium dihydrogen phosphate	Sharlau Chemie SA
Sodium hydrogen carbonate	BDH Chemicals Ltd.
Sodium hydroxide	Merck Ltd.
Sulphuric acid	BDH Chemicals Ltd.
Tetrabutylammonium bisulfate	Sigma Chemical Co.
Trichloroacetic acid (TCA)	Merck Ltd.
Uric acid	Sigma Chemical Co.
Xylenol orange	Sigma Chemical Co.

2.4.2 Muscle tissue homogenization procedure for oxidation products and loss of tissue antioxidants

Samples were ground to a fine powder in a mortar and pestle under liquid nitrogen and weighed into 1.8 ml Nunc cryotubes. 1.5 ml of ice cold 10 mM KCl and 20 µl/ml each of 10 mg/ml EDTA and 20 mg/ml BHT in methanol were added to the tube. The solution was homogenized on medium high speed by a Heidolph DIAX 900 and the blade was rinsed in a small volume of KCl, which was pooled with the tissue homogenate and made up to a total volume of 1.8 ml. This was stored at -80 °C.

2.4.3 Muscle tissue homogenization procedure for creatine compounds, nucleosides, nucleotides and related bases (PCA extraction)

Samples were ground and weighed as above. 1.5 ml of ice cold 0.4 M perchloric acid (PCA) was added to the ground tissue and the solution was homogenized. The blade was rinsed in a small volume of PCA, pooled with the sample and the volume made up to 1.8 ml. This was centrifuged at 500 rpm for 5 minutes. 500 µl of the supernatant was removed, neutralized to pH 7.0 with 55 µl of 2 M K₂CO₃ and stored at -80 °C.

2.4.4 Spectroscopic measurement of protein carbonyl concentrations

Protein carbonyls were derivatised with 2,4-dinitrophenyl hydrazine (DNPH) and measured by spectroscopy using the technique of Quinlan et al. (1994). Two 100 µl aliquots of sample were taken and placed in glass screw top tubes. 1 ml of 10 mM DNPH

in 2 M HCl was added to the first and 1 ml of 2 M HCl was added to the second making an individual control for each sample. These were then incubated with gentle mixing at 37 °C for 90 minutes. After incubation, 1 ml of 28% trichloroacetic acid (TCA) was added to each tube, which was then placed on ice for 5 minutes and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the protein pellet was washed in 5 ml of 50% ethanol 50% ethyl acetate and centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded and the washing step repeated. The tubes were left upside down for 5 minutes to drain before the protein pellet was dissolved in 1 ml of 6 M guanidine hydrochloride in 2 M HCl. This was left in the dark for a further 60 minutes to allow colour development. Absorbencies at 360 nm were measured using a UV-1601PC spectrophotometer supplied by Shimadzu NZ Ltd. Protein carbonyl concentrations were calculated by subtracting the paired control tubes from the DNPH derivatized tubes and dividing by an extinction coefficient of $21\,000\text{ l M}^{-1}\text{ cm}^{-1}$ for the protein carbonyl-DNPH complex.

2.4.5 Fluorescence detection of isoeugenol concentrations

Isoeugenol was solubilized into ethanol and quantified by fluorescence (Janssen unpublished). 100 µl of tissue homogenate was mixed with 900 µl of cold ethanol and vortexed for 30 seconds. This was placed on ice for 15 minutes and then centrifuged at 10 000 rpm at 4 °C for 10 minutes. 500 µl of the supernatant was placed into 0.5 ml quartz cuvettes and measured in a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA) with an excitation wavelength of 266 nm and an emission wavelength of 340 nm. Isoeugenol concentrations were determined by comparison to standards of known concentration.

2.4.6 HPLC analysis of TBARS concentrations

TBARS were derivatized with 2-thiobarbituric acid (TBA), separated by high performance liquid chromatography (HPLC) and quantified with fluorescence detection (Agarwal and Chase 2002). 100 µl of thawed tissue homogenate was mixed with 50 µl of 150 mM phosphoric acid, 50 µl of 42 mM TBA and 10 µl 20 mg/ml BHT in methanol and incubated at 95 °C for 30 minutes with gentle mixing to allow formation of the TBA-MDA complex. The samples were cooled on ice and centrifuged at 10 000 rpm at 4 °C for 10 minutes. 50 µl of the supernatant was removed and mixed with 200 µl of ice cold

methanol. This was placed on ice for 5 minutes and then centrifuged a second time. 10 μ l of supernatant was injected onto the HPLC.

The HPLC was a Shimadzu SIL-10A system supplied by Shimadzu NZ Ltd. The column was a reversed phase 5 μ m C18 150 x 4.6 mm column and was protected with a C18 SecurityGuard guard column. Both were supplied by Phenomenex Inc. The column was maintained at 30°C. The mobile phase was isocratic and consisted of 55% 50 mM NaH₂PO₄ adjusted to pH 6.8 with 10 M NaOH and 45% methanol pumped at 1 ml/min. Fluorescence detection with an excitation wavelength of 525 nm and an emission wavelength of 550 nm was used to detect the TBA-MDA complex. Peak areas were calculated using a Shimadzu software package and sample concentrations were determined by comparison to standards of known concentration.

2.4.7 HPLC analysis of ascorbic acid and uric acid concentrations

Ascorbic acid and uric acid were separated by ion-pair liquid chromatography on a Shimadzu SIL-10A HPLC system and quantified with electrochemical detection (Levine et al. 1999). The column was a reversed phase 5 μ m C18 250 x 4.6 mm column protected with a C18 SecurityGuard guard column both supplied by Phenomenex Inc. The column was maintained at 30°C. The mobile phase consisted of 40 mM sodium acetate, 0.54 mM EDTA, 0.5 mM dodecyltrimethylammonium chloride and 7.5% methanol adjusted to pH 4.8 with glacial acetic acid. This was run isocratic at 1 ml/min and the column was allowed to equilibrate in mobile phase for 16 hours before analytical work began.

Samples were centrifuged at 10 000 rpm and 50 μ l of supernatant was taken and mixed with 200 μ l of 90% methanol 10% 10 mM EDTA. This was placed on ice for 5 minutes to allow protein precipitation and then centrifuged at 10 000 rpm. 20 μ l of the supernatant was injected onto the HPLC. Electrochemical detection at +0.6 V was used to quantify ascorbic and uric acids. Peak areas were calculated with a Shimadzu software package and compared to standards of known concentration.

2.4.8 HPLC analysis of vitamin E concentrations

Vitamin E (RRR- α -tocopherol) was extracted into hexane, dried under oxygen free nitrogen and dissolved in methanol, separated by HPLC and quantified with fluorescence detection (Teissier et al. 1996). 100 μ l of sample was diluted with 400 μ l of water in screw top glass tubes. 10 μ l of 100 mg/ml EDTA, 25 μ l, 20 mg/ml BHT in methanol, 500 μ l of

ice cold ethanol and 1 ml of cold hexane was added to the tubes and the tubes vortexed for 60 seconds. They were then centrifuged at 4 000 rpm for 5 minutes to separate the phases. 600 μ l of the upper hexane layer was transferred into tapered bottom glass tubes and dried under oxygen free nitrogen in a warm water bath. The residues were dissolved in 100 μ l of cold methanol and 20 μ l was injected onto the HPLC.

The HPLC was a SIL-10A system supplied by Shimadzu NZ Ltd. The column was a reversed phase 5 μ m C18 125 x 4 mm column supplied by Alltech Associates Inc. and was maintained at 35°C. The mobile phase consisted of 100% HPLC grade methanol pumped at a rate of 1 ml/min. Detection was carried out by fluorescence with excitation at 292 nm and emission at 335 nm. Peak areas were calculated with a Shimadzu software package and compared to standards of known vitamin E concentration.

2.4.9 HPLC analysis of glutathione concentrations

Glutathione was derivatized with the fluorescent dye monobromobimane (MBB), separated by HPLC and quantified with fluorescence detection (Cotgreave and Moldeus 1986).

100 μ l of supernatant from the PCA extracted muscle tissue was placed in an Eppendorf tube. 5 μ l of 0.1 M NaOH was added to give a pH of approximately 8.0 followed by 5 μ l of 40 mM MBB dissolved in acetonitrile. This was mixed and left in the dark at room temperature for 20 minutes. After the incubation period, the tubes were centrifuged at 10 000 rpm for 5 minutes and 10 μ l of the supernatant was injected onto the HPLC.

The HPLC was a SIL-10A system supplied by Shimadzu NZ Ltd. The column was a reversed phase 5 μ m C18 150 x 4.6 mm column maintained at 30°C and protected with a C18 SecurityGuard guard column both supplied by Phenomenex Inc. The mobile phases consisted of 0.25% acetic acid (phase A) and 100% HPLC grade acetonitrile (phase B). These were run at 1.5 ml/min for 10 minutes at 90% phase A and 10% phase B, followed by a 1 minute gradient to 100% phase B. This was maintained for a further 4 minutes before the initial conditions were restored with a 1 minute gradient back to 90% phase A and 10% phase B. The column was allowed to equilibrate for 4 minutes before the next injection, giving a total run time of 20 minutes. Detection of the glutathione-MBB derivative was carried out by fluorescence with an excitation wavelength of 394 nm and an emission wavelength of 480 nm. Peak areas were calculated with a Shimadzu software package and compared to standards of known glutathione concentration.

2.4.10 HPLC analysis of creatine compounds, nucleosides, nucleotides and related bases

Creatine compounds, nucleosides, nucleotides and related bases were separated by ion-pair liquid chromatography and quantified by UV-Vis detection (Furst and Hallstrom 1992). The PCA extracted and neutralized muscle tissue samples were centrifuged at 10 000 rpm and 20 µl was injected onto the HPLC.

The HPLC was a SIL-10A system supplied by Shimadzu NZ Ltd. The column was a reversed phase 5 µm ODS (C18) 250 x 4.6mm column protected with a C18 SecurityGuard guard column both supplied by Phenomenex Inc. The column was maintained at 30°C. Buffer A consisted of 0.05 M NaH₂PO₄ and 2 g/l tetrabutylammonium bisulfate adjusted to pH 5.5 with 10 M NaOH. Buffer B was a mixture of 75% buffer A and 25% HPLC grade acetonitrile. The system was run at 1 ml/min in 100% buffer A for 5 minutes followed by a 20 minute gradient to 70% buffer B. This was held for 3 minutes followed by a 2 minute gradient back to 100% buffer A. The column was equilibrated in 100% buffer A for a further 20 minutes, giving a total run time of 50 minutes. Dual wavelength UV-Vis detection was carried out at 214 and 254nm to allow accurate quantification of creatine compounds and avoid the disturbance in base line caused by the solvent gradient. Peak areas were calculated with a Shimadzu software package and compared to standards of known concentrations.

2.5 Statistical analysis

Data was entered and managed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA). Unless otherwise stated all concentrations are expressed as nmol/g wet weight of tissue. Graphs and statistical analysis were produced using GraphPad Prism v4.03 (GraphPad Software Inc., San Diego, CA). All data is displayed as the mean ± the standard error of the mean (SEM). Data from the perfused tails was tested using paired students t-tests. As two samples were collected from each preparation samples were therefore not independent, and a paired students t-test was the most powerful and appropriate statistical test (McClave et al. 1997). Data from fillets stored at 15°C was tested using repeated measures two-way ANOVA with Bonferroni post-tests. This allowed simple analysis of the effects of both time and harvest type on each factor measured in these experiments (McClave et al. 1997). Significant interactions in the data sets indicate

that the trends observed during storage were modified by the harvesting procedures used. Data from fillets stored at 6°C was tested using one-way ANOVA with Dunnett's post-tests comparing storage means to the pre-storage mean. The threshold for statistical significance in each experiment was $p < 0.05$.

2.6 Method evaluation and development

2.6.1 Rested harvesting procedures

The chinook salmon used for this work were consistently very sensitive to visible or audible movement. This would produce a very strong escape response amongst all the animals in a tank and generally lasted 30-60 seconds. The severity of this response would destroy any attempt to harvest properly rested fish. For this reason the fish were moved from the open hatchery raceways into covered large circular tanks where our movement within the room could be hidden from them.

The concentration of AQUI-S™ and its delivery into the tank was also problematic. Younger, smaller fish (0.9-1.3 kg) responded well to concentrations of AQUI-S™ up to 25 ppm and were generally much easier to work with. They also recovered from anaesthesia rapidly and we had no fatalities during recovery. Larger harvest weight animals (2.5-3.5 kg) were very sensitive and would show signs of distress during the onset of anaesthesia, particularly above an AQUI-S™ concentration of 18 ppm. The individuals showing distinct signs of distress, especially during the latter stages of anaesthesia, needed to be promptly removed from the tank. Fatalities either in the late stages of anaesthesia or during recovery were not uncommon in these individuals. Adequate mixing of the tank water was also required at all times. Fish that were in a deep state of anaesthesia and could be handled without response, still showed strong sensitivity to inhaling high concentrations of AQUI-S™, responding with a strong escape response.

For these reasons a degree of control and discretion was required to harvest fish in a rested state. The tank was brought up to an initial AQUI-S™ concentration of 14-15 ppm over 20 minutes. The behaviour of the fish was noted and the concentration increased to 17-19 ppm depending on behaviour. Fish that showed signs of distress such as periodic escape responses were removed from the tank as the anaesthesia progressed. Only individuals that had shown no signs of distress during anaesthesia, were unresponsive

to handling, and had ventilation rates approximately $\frac{1}{3}$ of normal were used for experimental work on any particular day.

2.6.2 Protein hydroperoxides

Protein hydroperoxides can be reduced by Fe^{2+} to produce Fe^{3+} , which can then be reacted with xylenol orange to form a complex and measured with spectroscopy at 560 nm. This technique was first developed for the measurement of lipid hydroperoxides by Wolff (1994) and later adapted for protein hydroperoxides (Gay et al. 1999; Gay and Gebicki 2002). I evaluated this method for use on muscle tissue homogenates and perfusion saline samples. Samples assayed using both the PCA and H_2SO_4 versions of this technique showed very high variability of up to 50% and low reproducibility. It was later found that the brand of chloroform used during this assay was important for reproducibility, although this knowledge still did not resolve these issues to an acceptable level. I was unable to produce a reliable concentration curve by assaying varying quantities of tissue homogenate. I was able to increase protein hydroperoxide concentrations in the perfusion saline used to perfuse salmon tails incubated with the radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37°C for 16 hours. The saline contained bovine serum albumin (BSA), which was oxidized by the AAPH treatment to produce the protein hydroperoxides. However, this remained the only successful application of this technique and was not useful as far as future work was concerned as that would predominantly involve homogenized tissue samples. At this time it was also discovered that assaying protein carbonyl concentrations in muscle tissue homogenates produced reliable and reproducible data. The reasons why the hydroperoxide technique did not function under these circumstances is not entirely clear. However it is likely that the process of producing a tissue homogenate and the complexity and uncontrolled nature of the chemistry in such a solution mean that the relatively unstable protein hydroperoxides were not effectively preserved. Also, a number of contaminants normally present in biological samples can interfere with the chemistry of the assay procedure itself. A number of these problems are discussed in Gay and Gebicki (2003) and their methodology is shown to effectively minimize interference. After this experience we felt that persevering with and troubleshooting this technique was unlikely to be beneficial for this research considering the much higher quality data being collected from other techniques.

Given the data shown by Gay and Gebicki (2003), I still consider it possible to utilize and adapt this technique for use with tissue homogenates if required.

2.6.3 TBARS

The TBARS assay has been used in many different forms as a measure of lipid peroxidation and its applications, reliability and shortcomings are discussed in detail in Del Rio et al. (2005). Despite much criticism these measurements have repeatedly been shown to be important indicators in the onset of a number of pathologic conditions such as atherosclerosis and cancer (Del Rio et al. 2005) and also for processed products in the meat industry (Raharjo and Sofos 1993).

The method used in our laboratory involved the reaction of samples with TBA in an acidic environment at 95°C and quantification with an HPLC based assay with fluorimetric detection. The mobile phase originally contained 70% 0.1 M NaH₂PO₄ pH 6.8 and 30% methanol. The quantity of phosphate in this buffer regularly created phosphate buildup around the plumbing connections and in the detector cell of our HPLC. To try to reduce this problem I experimented with mobile phases with a range of phosphate concentrations from 0 through to 0.1 M. It was found that reducing the phosphate concentration to 0.05 M and increasing the methanol content to 45% gave the best balance of clean reproducible chromatography while substantially reducing the phosphate running through the machine. This method was most similar to that outlined by Agarwal and Chase (2002). This reduction noticeably reduced the rate of phosphate precipitation in the HPLC.

Because the proteins in our sample material were not precipitated prior to the TBA reaction, this method included MDA that was covalently bound to proteins. Although this method has been criticized because TBA may react with other compounds in the sample during the TBA reaction, and that the high derivatization temperature may induce further oxidation and/or the breakdown of hydroperoxides to MDA (Del Rio et al. 2005), much of the MDA was likely to be protein bound and therefore it was necessary to include it in the TBA reaction. The addition of BHT prior to the TBA reaction in our method inhibits further oxidation from occurring, but may not have prevented interference from other compounds. This decision however created problems with protein contaminating the analytical column. Although the guard columns provided good protection for the column itself they would rapidly block over the course of a day. As the MDA released by lipid peroxide breakdown is highly reactive and many lipids are protein associated, we wished

to include the protein matrix in the TBA reaction. Therefore we decided that any attempts to purify the sample should therefore take place after the TBA reaction. I attempted to precipitate the proteins with both PCA and TCA. Although these treatments did not interfere with the chromatography of the standard solutions the TBA-MDA adduct was no longer present on the chromatograms of tissue homogenates. I then attempted to use C18 solid phase extraction (Phenomenex Strata-X SPE) to remove the proteins from the sample material. Again, this procedure worked for standard solutions but the TBA-MDA complex in tissue homogenates moved through the C18 cartridge too slowly to be recovered in a known and reproducible volume of solvent. At this stage I noticed that standard solutions dissolved in methanol produced slightly larger and cleaner peaks. For this reason I tried mixing 50 μ l of tissue homogenate with 200 μ l of ice cold methanol. This was placed on ice for 5 minutes and centrifuged at 10 000 rpm at 4°C for 10 minutes. Although many proteins are soluble in methanol, some precipitation from the sample material was evident. 10 μ l of the supernatant was injected onto the HPLC. Although this did not completely eliminate the slow rise in pressure caused by protein contamination it did reduce the rate significantly. This solution, although requiring regular changing of the guard column, was found to offer the best balance of sample preparation time and cost. This method of sample preparation was later found to be similar to that used by Wong et al. (1987). Many of these issues are discussed in Agarwal and Chase (2002), who recommended an *n*-butanol liquid extraction of the TBA-MDA complex to avoid protein contamination of the analytical column. This technique may be worth utilizing in the future if blocking of the guard column begins to take place at unacceptable rates. Any future developments for the measurement of MDA in biological samples should be closely monitored.

2.6.4 Creatine compounds, nucleosides, nucleotides and related bases

For simultaneous measurement of ATP related compounds there are two often utilized HPLC based techniques. The most frequently used technique in seafood and meat industry research was developed by Ryder (1985). Although this technique is fast and simple it only yields information on the ATP related compounds used for calculating K-values and does not allow the measurement of other important compounds involved in muscle metabolism such as phosphocreatine and other related oxidation products such as uric acid. Although more time consuming and complex we decided to use the method developed by Furst and Hallstrom (1992), which allowed simultaneous determination of all related

compounds. Initial attempts to replicate their methodology failed to produce reliable and reproducible chromatography and phantom peaks began to appear during blank injections. We examined the quality of the chemicals we were using and identified the ion-pairing reagent 97% tetrabutylammonium bisulfate supplied by Sigma-Aldrich Inc. as a likely source of contamination. We sourced a higher purity reagent made by Fluka but supplied by Sigma-Aldrich Inc. specifically for ion-pair chromatography. This immediately resolved the issue. To limit the precipitation risks posed by the original 0.1 M NaH_2PO_4 buffer we reduced the buffer concentration to 0.05 M. The chromatography remained reproducible following this change.

The original work by Furst and Hallstrom (1992) monitored absorbance at 214 nm. Although this allowed detection of all the compounds of interest the baseline was significantly disturbed by the solvent gradient. Monitoring at 254 nm avoided this disturbance, but did not allow detection of creatine, creatinine and creatine phosphate. Therefore dual wavelength monitoring at both 214 nm and 254 nm was used. Chromatograms showing the clean separation and successful detection of all creatine compounds, nucleosides, nucleotides and related bases in rested and exhausted white muscle tissue are shown in Figures 5.2 and 5.3.

2.6.5 Valine hydroperoxides

We attempted to use the method of Fu et al. (1995) to separate and quantify valine hydroperoxides by HPLC with the addition of a post column reaction. A secondary mobile phase containing 0.1 M Na_2CO_3 , 17.1 mM 2-mercaptoethanol and 18.6 mM *O*-phthalaldehyde (OPA) was pumped into the post column flow and into a reaction coil maintained at 30°C at a rate of 0.5 ml/min. Although the three derivatized valine hydroperoxides shown in Fu et al. (1995) could be easily detected using fluorescence in 1 mM valine standards previously exposed to ^{60}Co , they could not be sufficiently separated from the background noise in hydrolyzed BSA following the same treatment. Because purification of the samples would be required for this technique to work it did not offer any additional benefits over the technique used by Fu et al.(1995). Given its time consuming nature and the uncertainty that it could be successfully applied to muscle tissue homogenates it was decided to continue with better characterized and less time consuming techniques to measure protein oxidation.

3 - Oxidation occurs in the red muscle, but not the white muscle of *in vitro* perfused chinook salmon tails.

3.1 Introduction

Recently, the extension of muscle cell viability in fish products through supporting metabolism has been examined as a means of preserving tissue (Black 2002; Janssen 2003). These studies have shown the ability to partially maintain elements of tissue physiology and biochemistry in teleost white muscle. However, even with intervention, once removed from the animal the tissue eventually loses its ability to maintain cellular homeostasis resulting in the loss of cellular viability and the onset of autolysis. It has been suggested that supporting metabolism through the delivery of oxygen to the tissue may result in oxidative damage, particularly to the mitochondria, eventually resulting in cell death (Black 2002; Janssen 2003). The aim of this work was firstly to determine if oxidative damage was occurring in the red and white muscle of the perfused chinook salmon tail preparation. Cut surface pH has been widely used as an indicator of tissue quality and metabolic state in fish (Sigholt et al. 1997; Jerrett and Holland 1998; Jerrett et al. 2000; Kiessling et al. 2004; Olsen et al. 2006; Bosworth et al. 2007) and was used in this work to allow comparison. Tissue TBARS concentrations provided a measure of lipid oxidation and protein carbonyl concentrations a measure of protein oxidation. Secondly, we aimed to determine if oxidation is directly related to losses of the key tissue antioxidants ascorbic acid and vitamin E, and thirdly to determine if addition of the cheap and readily available antioxidants ascorbic acid and uric acid to the perfusion saline can be used to control oxidation within the perfused tissue.

3.2 Materials and Methods

Detailed methodology for this work is included in Chapter 2. Five groups of rested chinook salmon were used for these experiments. They were transported live to the University of Canterbury, where they were pithed and then 1 ml of heparinized saline was injected into the dorsal aorta through the roof of the mouth. The tail section of the fish immediately posterior to the dorsal fin was dissected away from the body leaving a 20 mm

section of exposed spinal column protruding from the preparation. The tail was weighed and placed in a temperature controlled bin at $15 \pm 0.5^\circ\text{C}$. Cannulas were inserted into the dorsal aorta and the caudal vein. Standard oxygenated freshwater teleost saline (Table 2.1) was pumped into the tail through the dorsal aorta at a rate of $2.5 \text{ ml min}^{-1} 100 \text{ g}^{-1}$ tail weight.

A control group ($n=4$) with a mean weight of $1331 \pm 78 \text{ g}$ was prepared as described for the perfused preparations and was placed in a temperature controlled bin at $15 \pm 0.5^\circ\text{C}$ but was not perfused. Two groups (both $n=4$) were perfused for a total of 5 hours, one with oxygenated freshwater teleost saline and the second with oxygenated freshwater teleost saline that contained $400 \mu\text{M}$ uric acid and $100 \mu\text{M}$ ascorbic acid. These groups had mean weights of $989 \pm 55 \text{ g}$ and $966 \pm 26 \text{ g}$ respectively. Two further groups were both perfused with standard teleost saline for a total of 10 hours. One of these groups was stimulated for 30 seconds with 2 mA currents administered at a frequency of 1 Hz every 30 minutes of perfusion time. The current was delivered through the spinal column by placing a 24 gauge hypodermic needle into the spinal column from the anterior end of the preparation and an alligator clip in the middle of the caudal fin. These were attached to a current generator (model CS200, J.P Trading, Aarhus, Denmark). The control group ($n=4$) had a mean weight of $815 \pm 103 \text{ g}$ and the stimulated group ($n=3$) had a mean weight of $857 \pm 279 \text{ g}$.

Samples were taken from the red and white muscle tissue before and after perfusion from undisturbed locations as specified in Janssen (2003). They were immediately freeze clamped and stored at -80°C for extraction.

Tissue samples were ground to a fine powder under liquid nitrogen, weighed and homogenized in 10 mM KCl with 20 $\mu\text{l/ml}$ each of 10 mg/ml EDTA and 20 mg/ml BHT in methanol. They were made up to a final volume of 1.8 ml and stored at -80°C for analysis.

TBARS were measured by derivatization with thiobarbituric acid, separation by reverse phase HPLC (Shimadzu SIL-10A system) and quantification by fluorescence (Agarwal and Chase 2002). Samples were diluted 1:5 in ice cold methanol and centrifuged at 10 000 rpm before injection. Protein carbonyls were derivatized with 2,4-DNPH and measured using spectroscopy (Quinlan et al. 1994). Ascorbic acid and uric acid were separated with ion pair reverse phase HPLC and quantified with electrochemical detection (Levine et al. 1999). Vitamin E was extracted into hexane, dried under nitrogen and dissolved in methanol. This was separated by reverse phase HPLC and vitamin E detected by fluorescence (Teissier et al. 1996).

3.3 Results

3.3.1 The effects of added antioxidants on protein and lipid oxidation occurring in *in vitro* perfused salmon tails.

As cut surface pH is indicative of metabolic state, it was used in this section to indicate perfusion performance. Cut surface pHs (Figure 3.1) from all three groups of tails reduced from around 7.6 to 7.3 over the 5 hour time period. This reduction was statistically significant ($p < 0.01$) for the unperfused tails and the tails perfused with standard freshwater teleost saline. The difference between pre and post perfusion cut surface pHs in the tails perfused with freshwater teleost saline containing the antioxidants uric acid and ascorbic acid was not statistically significant, potentially inferring improved performance. Closer examination of the data shows that the standard error was larger for this group and all three groups had small n values ($n=4$).

Pre treatment TBARS concentrations were 10-15 nmol g⁻¹ higher in the lipid rich red muscle than the white muscle. A statistically significant rise in TBARS concentrations of 10 and 13 nmol g⁻¹ respectively was shown in the red muscle of both tails perfused with standard saline and tails perfused with saline containing antioxidants (Figure 3.2). TBARS concentrations did not change in the red muscle of the unperfused tails. The white muscle tissue did not show any changes in response to the treatments (Figure 3.3).

Due to experimentation with analytical techniques during this work protein carbonyls, ascorbic acid and uric acid measurements were only made on the perfused groups. Vitamin E measurements were only made on the group perfused with freshwater teleost saline containing ascorbic and uric acids.

Protein carbonyls increased significantly in the red muscle of salmon tails perfused with oxygenated saline (Figure 3.4). The increase was slightly smaller in the red muscle of tails perfused with oxygenated saline containing ascorbic and uric acids and this change was marginally outside of statistical significance ($p=0.054$). Larger n-values would likely produce a statistically significant difference for this group. Pre treatment protein carbonyl concentrations were almost two fold higher in white muscle than in red muscle, but did not change significantly (Figure 3.5).

Pre-perfusion ascorbic acid concentrations in the white muscle were approximately 54 nmol g⁻¹ compared to 18 nmol g⁻¹ in the red muscle. Perfusion with standard teleost saline had no effect on ascorbic acid concentrations in either the red or the white muscle (Figures 3.6 and 3.7). Perfusion with saline containing ascorbic acid resulted in a

significant mean increase in ascorbic acid concentrations in the red muscle ($p < 0.05$). No changes were seen in the white muscle, indicating that the perfusion of the red muscle is far better than the white.

Very little or no uric acid was detectable in either the red or the white muscle tissue of the pre-perfusion salmon tails. The red muscle perfused with saline containing uric acid showed a very significant ($p < 0.001$) mean increase of 57 nmol g^{-1} , while uric acid remained undetectable in the red muscle following standard perfusion (Figure 3.8). A very small amount of uric acid was found in the white muscle following perfusion with uric acid but this change was not significant (Figure 3.9). This provides further evidence that the perfusion of the red muscle was much better than that of the white.

Mean pre-perfusion vitamin E concentrations were far higher in the lipid rich red muscle than in the white (Figures 3.10 and 3.11). There were no significant changes in vitamin E concentration in either the red or white muscle after perfusion with saline containing ascorbic and uric acids.

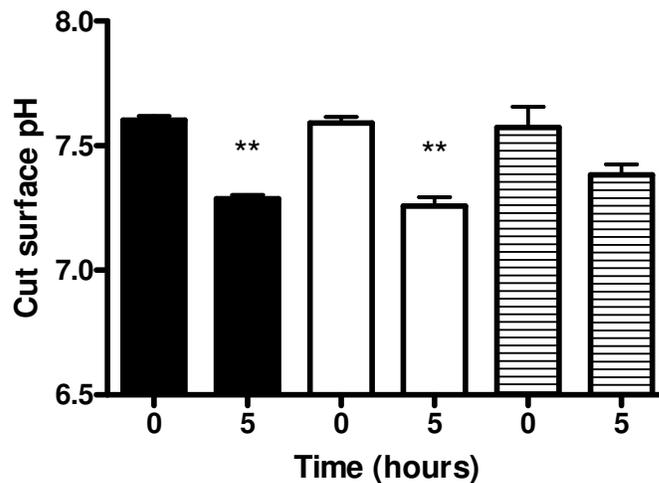


Figure 3.1 Mean cut surface pHs ($n=4$) from the white muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black), salmon tails perfused with oxygenated freshwater teleost saline (white) and salmon tails perfused with oxygenated freshwater teleost saline containing $100 \mu\text{M}$ ascorbic acid and $400 \mu\text{M}$ uric acid (crossed). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (** $p < 0.01$).

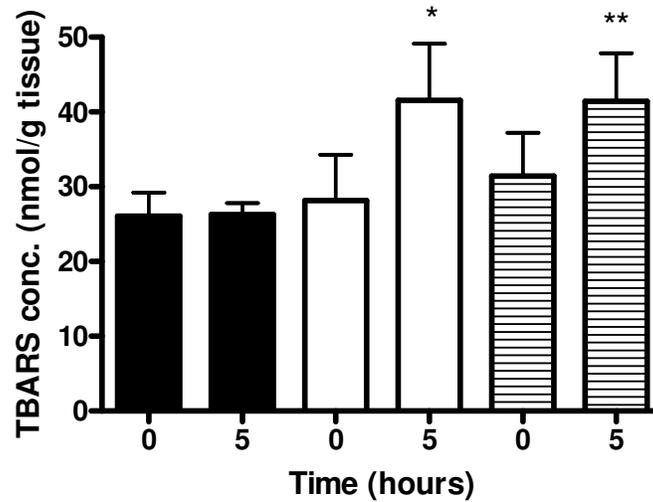


Figure 3.2 Mean TBARS concentrations from the red muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black, n=2), salmon tails perfused with oxygenated freshwater teleost saline (white, n=4) and salmon tails perfused with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed, n=4). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (* $p < 0.05$, ** $p < 0.01$).

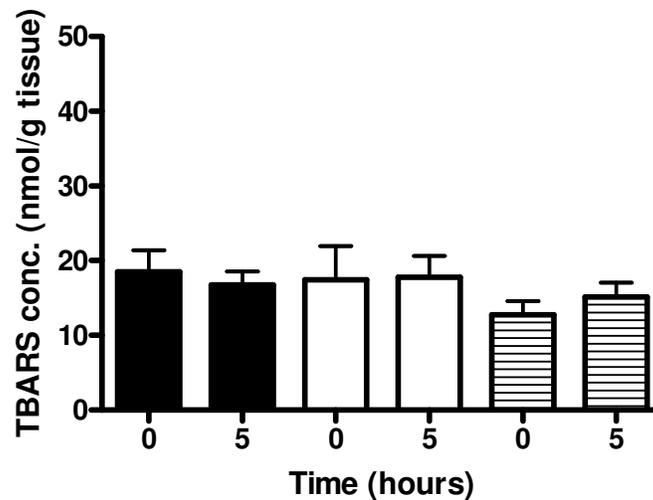


Figure 3.3 Mean TBARS concentrations (n=4) from the white muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black), salmon tails perfused with oxygenated freshwater teleost saline (white) and salmon tails perfused with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

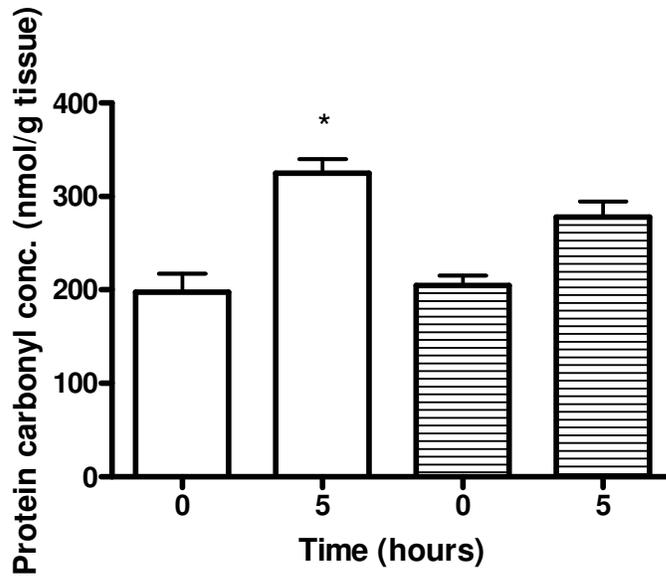


Figure 3.4 Mean protein carbonyl concentrations (n=4) from the red muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (* $p < 0.05$).

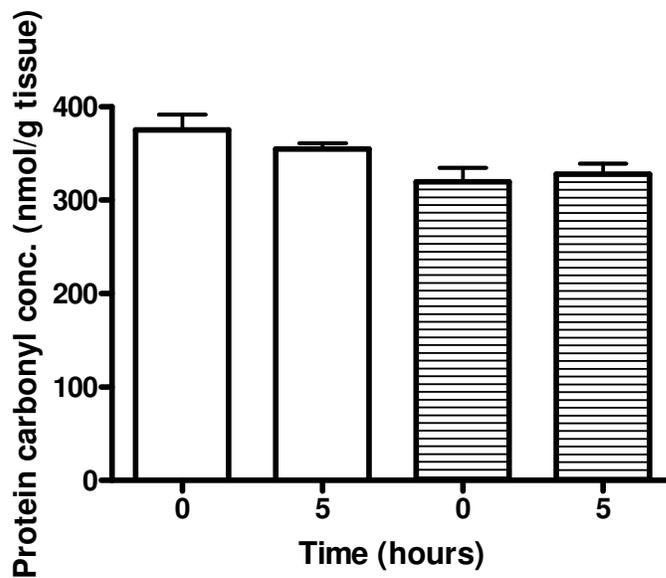


Figure 3.5 Mean protein carbonyl concentrations (n=4) from the white muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

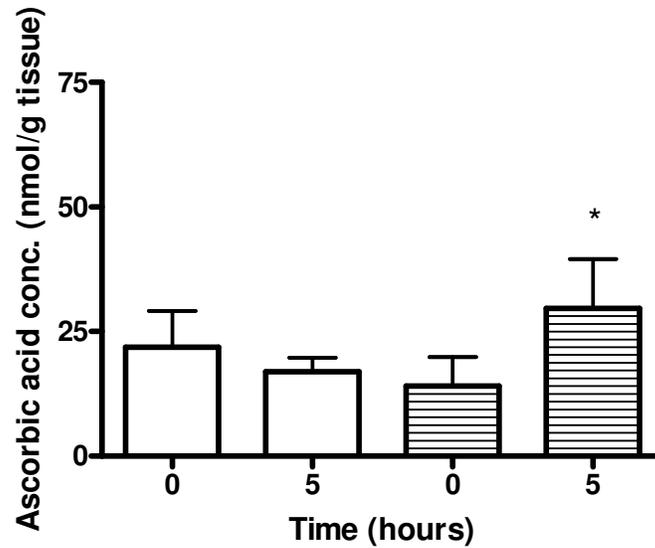


Figure 3.6 Mean ascorbic acid concentrations (n=4) from the red muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 µM ascorbic acid and 400 µM uric acid (crossed). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (* p<0.05).

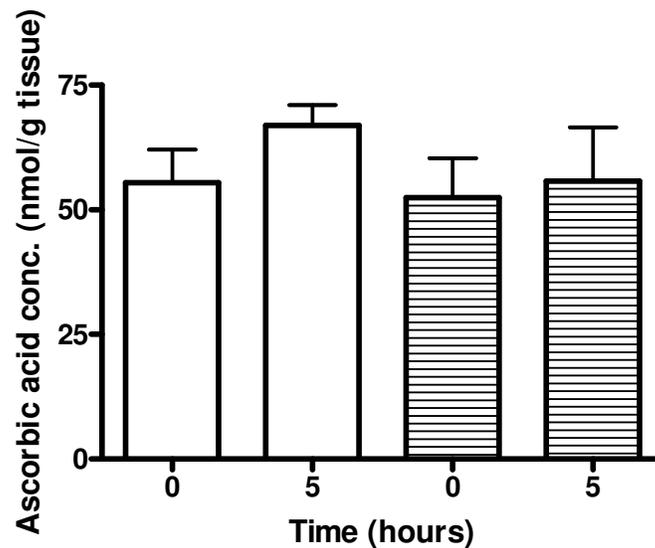


Figure 3.7 Mean ascorbic acid concentrations (n=4) from the white muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 µM ascorbic acid and 400 µM uric acid (crossed). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

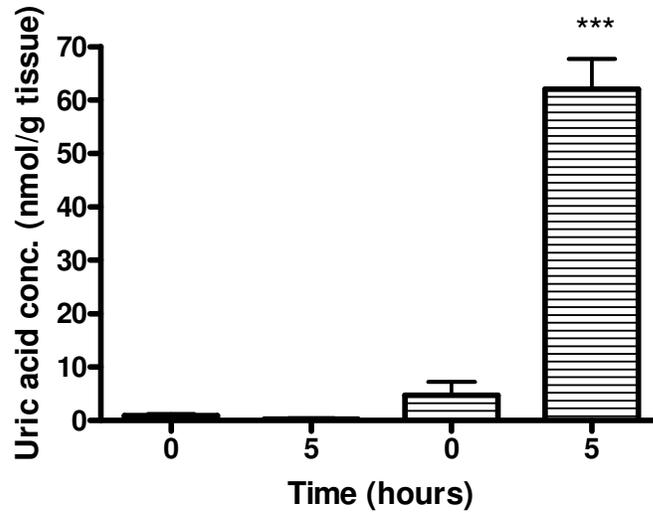


Figure 3.8 Mean uric acid concentrations (n=4) from the red muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (***) p<0.001).

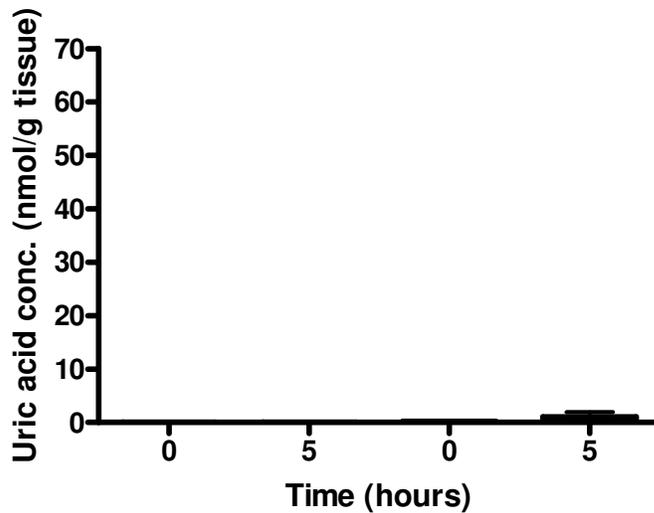


Figure 3.9 Mean uric acid concentrations (n=4) from the white muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white) and before and after perfusion with oxygenated freshwater teleost saline containing 100 μ M ascorbic acid and 400 μ M uric acid (crossed). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

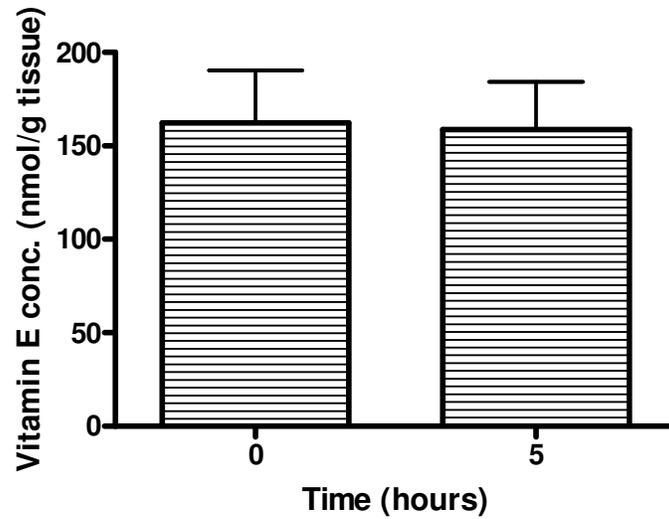


Figure 3.10 Mean vitamin E concentrations (n=4) from the red muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline containing 100 μM ascorbic acid and 400 μM uric acid. Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

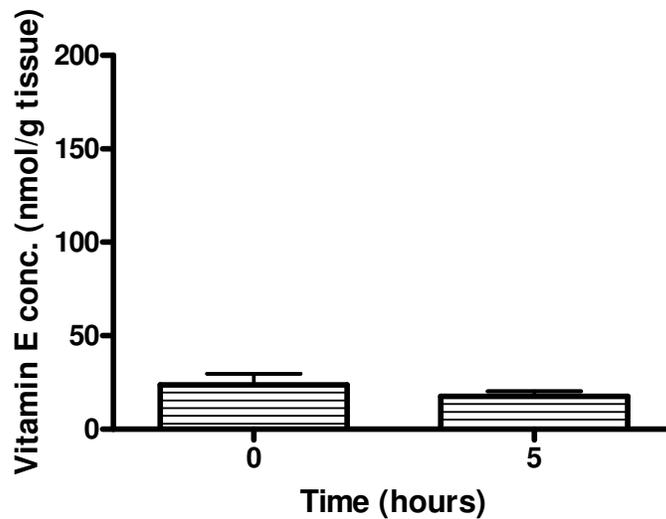


Figure 3.11 Mean vitamin E concentrations (n=4) from the white muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline containing 100 μM ascorbic acid and 400 μM uric acid. Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

3.3.2 The effects of electrical stimulation on lipid and protein oxidation in *in vitro* perfused salmon tails

As there was the potential for contracting muscles in the tail preparation to aid perfusion of the white muscle tissue, this experiment aimed to determine if increased perfusion of the white muscle tissue with oxygen saturated saline was sufficient to induce oxidation. Cut surface pH (Figure 3.12) reduced significantly in both the unperfused salmon tails and those perfused with oxygenated freshwater teleost saline for 10 hours. The tails that were perfused and received electrical stimulation showed a slightly smaller reduction than the other groups that was not significantly different from the pre-perfusion mean.

The change in TBARS concentrations in the red muscle following the three treatments was too small to attain statistical significance (Figure 3.13). TBARS concentrations reduced slightly in the white muscle of all three groups, but the changes were once again not statistically significant (Figure 3.14).

Protein carbonyl concentrations in the red muscle of both perfused groups showed similar sized increases following perfusion (Figure 3.15). The increase from the perfused group was statistically significant ($p < 0.05$), but the electrically stimulated group returned a p-value of 0.07. There were no changes in protein carbonyl concentration in the white muscle (Figure 3.16).

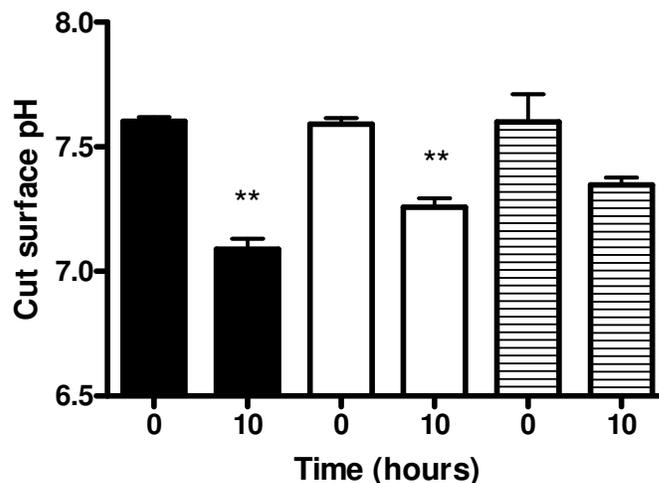


Figure 3.12 Mean cut surface pHs from the white muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black, $n=4$), salmon tails perfused with oxygenated freshwater teleost saline (white, $n=4$) and salmon tails perfused with oxygenated freshwater teleost saline and stimulated with a 2 mA current at a frequency of 1 Hz for 30 seconds every 30 minutes (crossed, $n=3$). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (** $p < 0.01$).

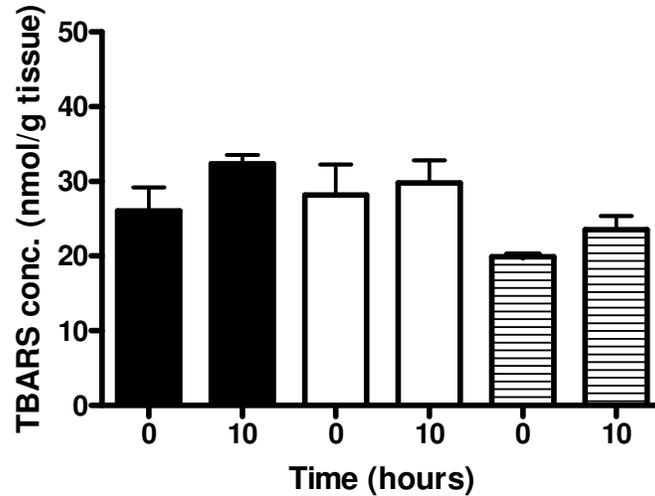


Figure 3.13 Mean TBARS concentrations from the red muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black, n=2), salmon tails perfused with oxygenated freshwater teleost saline (white, n=4) and salmon tails perfused with oxygenated freshwater teleost saline and stimulated with a 2 mA current at a frequency of 1 Hz for 30 seconds every 30 minutes (crossed, n=3). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

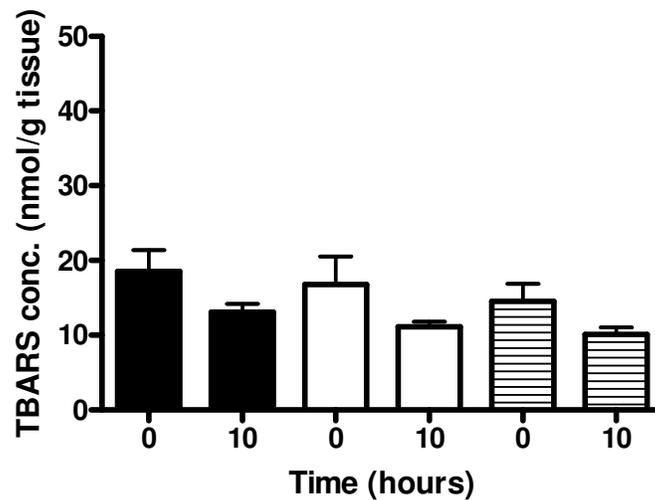


Figure 3.14 Mean TBARS concentrations from the white muscle of salmon tails before and after treatment. The three groups contained unperfused salmon tails (black, n=4), salmon tails perfused with oxygenated freshwater teleost saline (white, n=4) and salmon tails perfused with oxygenated freshwater teleost saline and stimulated with a 2 mA current at a frequency of 1 Hz for 30 seconds every 30 minutes (crossed, n=3). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

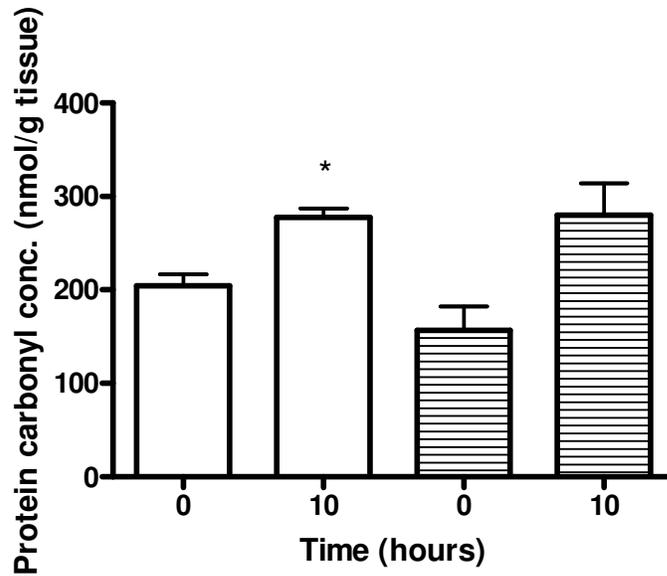


Figure 3.15 Mean protein carbonyl concentrations from the red muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white, n=4) and before and after perfusion with oxygenated freshwater teleost saline and stimulation with a 2 mA current at a frequency of 1 Hz for 30 seconds every 30 minutes (crossed, n=3). Error bars indicate SEM. The results of paired t-tests comparing pre and post treatment means are indicated (* p<0.05).

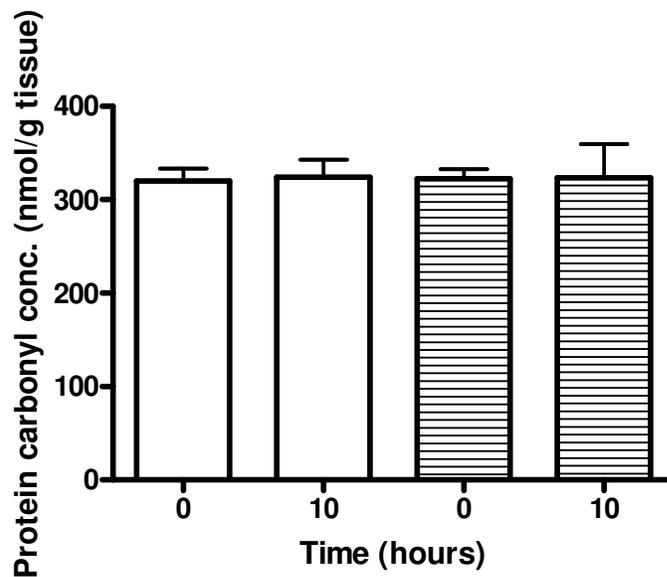


Figure 3.16 Mean protein carbonyl concentrations from the white muscle of salmon tails before and after perfusion with oxygenated freshwater teleost saline (white, n=4) and before and after perfusion with oxygenated freshwater teleost saline and stimulation with a 2 mA current at a frequency of 1 Hz for 30 seconds every 30 minutes (crossed, n=3). Error bars indicate SEM. Paired t-tests comparing pre and post treatment means were performed on these data and no significant differences were found.

3.4 Discussion

Acidification of white muscle in fish has been shown in response to stressful harvesting techniques (Sigholt et al. 1997; Jerrett et al. 2000; Kiessling et al. 2004; Olsen et al. 2006; Bosworth et al. 2007) and progresses as harvested fillets age (Black 2002). As the white muscle functions predominantly through anaerobic glycolysis, cut surface pH measurements also correlate well to lactic acid and H^+ concentrations within the tissue (Black 2002; Janssen 2003) and can therefore be used as an indicator of metabolic state. The retention or maintenance of high cut surface pHs in an *in vitro* preparation can indicate the performance of that preparation in supporting either a normal or reduced metabolic (hypometabolic) state. In the 5 hour perfusion periods undertaken in this work, cut surface pHs were taken only from the white muscle as the cross section of the red muscle was too small to sample with the pH probe. Figure 3.1 shows that comparable decreased cut surface pHs were seen in all of the tail preparations, perfused and unperfused, and although the statistical analysis suggest a slight improvement in the tails perfused with added ascorbic acid and uric acid, the improvement is very small. The cut surface pHs of the white muscle tissue of perfused tails were not as effectively preserved as was shown by Janssen (2003). The higher perfusion temperature used in this work to increase reaction rates in the tissue and promote oxidative processes may have reduced the effectiveness of the perfusion in preserving high cut surface pHs. Any improvement seen by adding antioxidants to the perfusion saline was small and its physiological significance uncertain.

There were notable differences between the red and the white muscle tissue. None of the measurements taken in this work changed in the white muscle during perfusion. The inability to deliver the antioxidants to the white muscle indicates that the white muscle is poorly perfused compared to normal blood flow distribution in the whole animal (Janssen 2003) and therefore our ability to manipulate its properties through perfusion is also poor. A significant improvement will be required before the effects of perfusion and increased tissue antioxidants in this tissue can be properly examined.

In Figures 3.2 and 3.4, our markers of lipid (TBARS) and protein oxidation (protein carbonyls) increased in the red muscle in response to perfusion with oxygenated saline, but not in unperfused tails. The increase in TBARS indicates that the flow of oxygen saturated saline through the red muscle is causing oxidation that would not otherwise occur. Increases in oxidation products within tissues has been shown in response to stressful conditions in marine animals (Heise et al. 2006) and are indicative of a number of disease

pathologies in medicine (Shacter 2000; Del Rio et al. 2005). Therefore further investigation into the nature of the metabolic state in the red muscle during perfusion would be required to determine whether metabolism is being appropriately supported, as there is a danger that the oxidative stress placed on the tissue may outweigh any benefits gained from supporting metabolism.

There were noticeable differences in the red muscle between the 5 hour perfusions and the 10 hour perfusions shown in Figures 3.13 and 3.15. Protein oxidation of the red muscle at 10 hours was comparable to that at 5 hours and lipid peroxidation at 10 hours was less than that at 5 hours. This indicates that these products are labile and it is likely that they are being flushed out of the preparation by the perfusion saline. However any labile oxidation products in the perfusion saline had undergone far too large a dilution to be measurable.

We attempted to control the oxidation occurring within the red muscle by adding ascorbic acid and uric acid to the perfusion saline. This decision was made for two reasons. Firstly, they are both inexpensive and readily available antioxidants, and secondly, ascorbic acid has been shown to inhibit potential pro-oxidant effects of uric acid (Abuja 1999). There was a slight reduction in protein oxidation (Figure 3.4), but not lipid oxidation (Figure 3.2) seen in the tails perfused with saline containing added antioxidants. The physiological significance of this reduction is likely to be small however, especially given the high concentrations of the respective antioxidants shown in the red muscle following perfusion (Figures 3.6 and 3.8). This raises questions regarding the suitability of simple antioxidant solutions to provide complex tissues with protection from oxidation above that already provided by their own endogenous antioxidant systems. This may not be entirely unexpected considering that within normal biological systems, antioxidant systems function as complex interacting oxidation and reduction reactions, with antioxidant compounds being continually recycled (Buettner 1993; Packer et al. 1999). A number of studies have examined using both simple antioxidant solutions such as ascorbic acid (Richards et al. 1998; Hamre et al. 2003) and more complex plant extracts (Tozer 2001; Goulas and Kontominas 2007) to inhibit oxidation in seafood products. Reduced levels of oxidation were shown in some of these treatments, but unlike the current work these changes occurred outside the period in which normal metabolic processes would have been occurring due to the treatment types and/or the long time frames. Although the current work does not conclusively show that simple antioxidant solutions do little to inhibit oxidation and support metabolism in the perfused salmon tail model, because the

effect of varied antioxidant concentrations was not investigated, it seems unlikely that this approach would yield significant improvements. The use of plant extracts has the potential to deliver improvements in the perfusion due to the complex antioxidant systems this would introduce into the perfusion saline. As some plant extracts have vasoactive properties (Packer et al. 1999; Ghayur et al. 2005; Padilla et al. 2005) and have been shown to reduce oxidation in seafood products (Tozer 2001) there is the potential for improved perfusion and oxidative stability.

This work shows average tissue concentrations and does not provide further information regarding the location of the oxidized proteins and lipids or the antioxidants introduced into the tissue. Determining whether the oxidation is extracellular, caused by the high oxygen partial pressures introduced by the perfusion saline, or whether it is intracellular and connected to metabolic processes, particularly within the mitochondria which have been shown to generate reactive oxygen species (Nohl and Hegner 1978), would aid our understanding of the effectiveness of the perfusion preparation in supporting metabolism. The inability of uric acid to provide protection from oxidation may be at least partially caused by being unable to efficiently cross cell membranes, potentially isolating it from a major source of reactive oxygen species. For this reason lipid soluble antioxidants may prove a better choice. In rat livers, resveratrol has shown the ability not only to act as an efficient antioxidant but also to act directly on the mitochondria to preserve function (Plin et al. 2005). Compounds with modes of action other than strict function as an antioxidant have potential to further improve the performance of the tail preparation.

Comparison of TBARS concentrations in the red muscle measured in this work to that from other researchers is made difficult for two reasons. Firstly, spectroscopic TBARS measurements cannot be directly compared to the more specific HPLC based measurements remain widespread in the literature, and secondly the white muscle is of primary interest and oxidation of the red muscle is therefore seldom reported. However Undeland (2001) showed that general markers of lipid oxidation are elevated in dark muscle compared to light muscle, which is in agreement with the data shown in Figures 3.2 and 3.3.

Despite the level of lipid oxidation observed in the red muscle there was no decrease in tissue vitamin E concentrations (Figure 3.10), although it must be noted that these measurements were only taken in the perfusions supplemented with antioxidants. Vitamin E has been shown to have both pro and antioxidant activity (Thomas et al. 1995; Kontush et al. 1996; Neuzil et al. 1997) and to interact with co-antioxidants such as

ascorbic acid (Lee and Dabrowski 2003). This makes determining the overall contribution of vitamin E to the oxidative stability of the red muscle difficult. Also, the anaesthetic used to prepare and transport the fish contains the lipid soluble antioxidant isoeugenol. There is the possibility that this contributed to the lipid soluble fraction of the tissue antioxidants and affected vitamin E concentrations: however Kadoma et al. (2006) showed that although eugenol has an ability to regenerate vitamin E, isoeugenol does not. It would seem that despite these confounding factors, lipid oxidation can occur in the tissue without necessarily depleting or reducing vitamin E concentrations first. The ability of vitamin E to act as an antioxidant has dominated much of the literature. More recently however a number of cellular functions that are independent of its antioxidant abilities have been reported (Azzi et al. 2004; Zingg and Azzi 2004). The current work suggests that the primary functions of vitamin E in salmon skeletal muscle may not be strictly related to its antioxidant function. There have been many studies showing that elevating vitamin E concentrations reduces lipid oxidation both *in vivo* and in tissues post-mortem (Schaefer et al. 1995; Jensen et al. 1998; Flader et al. 2003). However, vitamin E concentrations did not change in response to measurable lipid oxidation in the red muscle of this preparation and therefore further elevating tissue vitamin E concentrations either through pre-harvest diet or in an emulsion with the perfusion saline seems unlikely to increase the resistance of the red muscle to oxidation during perfusion.

This work clearly shows that the introduction of oxygen into the red muscle tissue of post-mortem salmon tails causes both protein and lipid oxidation in the tissue. We have not been able to link this oxidation to tissue antioxidant loss and our attempts to control it by adding the antioxidants ascorbic acid and uric acid to the perfusion saline had little protective effect. Introducing oxygen into post-mortem tissues may have the ability to temporarily support normal metabolic processes but clearly carries with it a risk of increased oxidative damage to the tissue that may not be easily controlled.

Electrical stimulation to the spinal column has been shown to improve blood flow through fish tails (Satchell 1964; Janssen 2003). The stimulation and increased perfusion time was an attempt firstly to improve perfusion of the white muscle, and secondly to allow more time for tissue properties to change. The electrical currents caused strong contractions through the preparations at the beginning of the perfusion period and reduced in their intensity until they were barely visible at 10 hours. Again, there were no significant changes in lipid or protein oxidation in the white muscle (Figures 3.14 and 3.16). Although we had no direct means of measuring perfusion of the white muscle, any

increase was still insufficient to cause oxidation within the tissue. In the red muscle (Figures 3.13 and 3.15), instead of increasing tissue oxidation there were no significant changes in TBARS concentrations and, although protein oxidation was evident, the changes were comparable to the 5 hour perfusions. This may indicate that the preparation spends some time reaching a relatively static metabolic state during perfusion given that the suitability of the sampling times chosen for this work is unknown. It would be very beneficial to generate a time course for the oxidation of the red muscle tissue in this perfusion model. However, this would be very time consuming as the preparation is destroyed in the process of sampling. Although the strength of the contractions in the stimulated preparation indicated exhaustion, the cut surface pH of the muscle shown in Figure 3.12 was relatively high. Given the correlation shown between muscle cut surface pH and muscle lactate concentrations (Black 2002; Janssen 2003), this indicates that lactate buildup was not a problem for the white muscle. It is likely that much of the lactate was flushed from the tissue by the perfusion saline and that the exhaustion was related more to the inability of the muscle to supply enough energy for contraction than contractile inhibition caused by lactate buildup and intracellular acidification. Given the data, it is difficult to reach any firm conclusions, although the electrical stimulation treatment does not improve oxygen delivery enough to initiate oxidation within the white muscle. The relatively high cut surface pHs measured despite severely reduced contractile ability indicate likely removal of metabolic waste products and therefore the perfusion is helping to preserve some aspects of tissue function.

Although nitric oxide (NO) has been shown to play an important role in the regulation of vascular tone in mammals (Torreilles 2001), its role in fish is less certain (Eddy 2005). As it reacts with superoxide to form ONOO⁻, its production *in vivo* contributes to the redox environment, has been shown to influence the tenderness of certain types of meat (Warner et al. 2005) and plays a role in reperfusion injury in skeletal muscle (Khanna et al. 2005). NO donors have been shown to vasodilate, and NO inhibitors vasoconstrict rainbow trout coronary systems (Mustafa et al. 1997) indicating that it does play a role in regulating vascular tone in salmonids. Attempts have been made in our laboratory to use the NO donator sodium nitroprusside to improve the perfusion of the white muscle in our perfused salmon tails, but these were unsuccessful (Janssen unpublished). Improved control of factors regulating vascular tone will be required to allow adequate perfusion of the white muscle in this tail perfusion model.

3.4.1 Summary

These data question our understanding of the metabolism and chemistry of the red muscle of the salmon tail preparation. A great deal of further work would be required to understand the metabolic processes and the progression of oxidation during perfusion. Controlling oxidation and maintaining the redox balance of the cells is likely to be important for long term maintenance of cell viability in perfusion models. However, it seems unlikely that adding simple commercially available antioxidants can contribute greatly. It is clear however that the white muscle is relatively stable in relation to the measurements that we have taken and that our ability to manipulate its properties through perfusion is limited. The white muscle is of primary importance commercially and therefore investigating its properties in relation to tissue oxidation and antioxidant depletion further using another experimental model seems appropriate.

4 - Rested harvesting with AQUI-S™ inhibits lipid oxidation in chinook salmon tail fillets.

4.1 Introduction

The aim of this chapter was to create a profile of the protein and lipid oxidation and the loss of the key antioxidants vitamins C and E in chinook salmon white muscle tissue during storage. This was primarily to address the concerns raised in Chapter 3 that there were no measurable increases in oxidation products or losses of tissue antioxidants in the white muscle tissue during perfusion of the tail. Therefore determining the time frames in which oxidation and the loss of tissue antioxidants were taking place and their relation to post harvest metabolic state and cell death was important. The tissue properties measured are the same as those measured in Chapter 3 and include protein carbonyls as a measure of protein oxidation, TBARS as a measure of lipid oxidation, and uric acid, vitamin C and vitamin E as indicators of tissue antioxidant status.

4.2 Materials and Methods

Detailed methodology for this section can be found in Chapter 2. Three groups of harvest weight chinook salmon were used to determine the effects of rested harvesting on tissue oxidation and determine any tissue properties related to isoeugenol exposure and not to rested tissue. The first group (n=4) was harvested using rested harvesting procedures and had a mean weight of 3195 ± 190 g. The second group (n=4) was exhausted during Isaac Salmon's normal harvesting procedures (mean weight of 3133 ± 406 g) and the third (n=4) was exhausted and then placed through the rested harvesting procedures (mean weight of 2170 ± 141 g). The fish were killed by cranial impact, bled and transported on ice to the University of Canterbury for fillet preparation.

Fillets were prepared from the left side of the fish beginning at the dorsal fin and running to the tail. The cut surface pH of the fillet was taken as the mean of six concurrent measurements from the D block, three each side of the spine beginning 10 mm from the anterior end of the fillet. Samples were taken from the D block and freeze clamped and stored at -80°C for extraction. The fillets were placed in a temperature controlled bin at $15 \pm 0.5^{\circ}\text{C}$. Repeated pH measurements and samples from undisturbed locations not

including the posterior third of the fillet were taken at 6, 12, 24, 30, 36, 48, 54, 60, 72 and 96 hour time points.

Samples were taken from both the exposed surface tissue and the unexposed tissue from the centre of the fillet for the exhausted group exposed to rested harvesting techniques. This was to determine the influence of air exposure on protein oxidation.

A further group of exhausted fish was used to determine the effects of temperature on the fillet preparations. This group (mean weight 2170 ± 141 g) was harvested and prepared as specified above but was placed in a temperature controlled bin at $6 \pm 0.5^\circ\text{C}$ and sampled at 2 day intervals for a total of 8 days.

Tissue samples were ground to a fine powder under liquid nitrogen, weighed and homogenized in 10 mM KCl with 20 $\mu\text{l/ml}$ each of 10 mg/ml EDTA and 20 mg/ml BHT in methanol. The homogenates were made up to a final volume of 1.8 ml and stored at -80°C for analysis.

TBARS were measured by derivatization with thiobarbituric acid, separation by reverse phase HPLC (Shimadzu SIL-10A system) and quantification by fluorescence (Agarwal and Chase 2002). Samples were diluted 1:5 in ice cold methanol and centrifuged at 10 000 rpm before injection. Protein carbonyls were derivatized with 2,4-DNPH and measured using spectroscopy (Quinlan et al. 1994). Ascorbic acid and uric acid were separated with ion pair reverse phase HPLC and quantified with electrochemical detection (Levine et al. 1999). Vitamin E was extracted into hexane, dried under nitrogen and dissolved in methanol. This was separated by reverse phase HPLC and vitamin E detected by fluorescence (Teissier et al. 1996).

4.3 Results

4.3.1 The effects of harvesting procedures on oxidation of the white muscle

As rested harvesting procedures produce salmon fillets in a rested physiological state that contain the lipid soluble antioxidant isoeugenol, our aim was to examine the progression of oxidative processes in rested tissues and compare these to exhausted tissues that are similar to those produced in the salmon farming industry. Three harvesting techniques were used to separate any potential antioxidant effects of isoeugenol from effects related to the rested metabolic state of the tissue.

Cut surface pHs from the rested harvest group began at 7.45 and reduced to 6.6 over 36 hours (Figure 4.1). The exhausted group and the exhausted group harvested with rested harvesting techniques had pre-storage mean cut surface pHs of 6.5 and 6.6 respectively, indicating their exhausted state. These reduced slightly to 6.4 over 36 hours. All 3 groups had inflection points at around 36 hours where the cut surface pH began to rise again, which appeared to coincide with the onset of rancidity indicated by a noticeable odour. Two-way ANOVA showed that there was a significant interaction between these data ($p < 0.001$) indicating that cut surface pH trends are dependent on harvesting technique.

White muscle TBARS concentrations in the exhausted group began to rise at about 36 hours and continued to rise until 96 hours (Figure 4.2). No rise was seen in either of the other two groups. There was no significant interaction between these data, but both time ($p < 0.01$) and harvesting technique ($p < 0.001$) were significant sources of variation. Although TBARS concentrations in the exhausted group began to rise at 36 hours, Bonferroni post-tests showed statistically significant differences occurred only at 72 and 96 hours.

Protein carbonyl concentrations rose consistently in all three groups, approximately doubling over 96 hours (Figure 4.3). No significant difference were found between any of the groups and time was the only significant source of variation ($p < 0.001$).

Tissue vitamin C concentrations showed a high degree of variability in all three data sets (Figure 4.4). In both groups harvested with AQUI-S™, vitamin C concentrations were high at the beginning of the experiment and were relatively stable for the first 24 hours before decreasing steadily approaching undetectable levels at 96 hours. The exhausted group had very low levels of vitamin C that increased over the first 6 hours before following a similar trend to the other two groups. Time was the only significant source of variation in these data ($p < 0.001$) and the pre-storage mean in the exhausted group harvested with AQUI-S™ was the only data point that was significantly different from the exhausted group.

Uric acid was undetectable in almost all samples taken from the two groups harvested using AQUI-S™ (Figure 4.5). The exhausted group accumulated uric acid from 36 hours finishing with a mean concentration of over 200 nmol g⁻¹. There was significant interaction in these data ($p < 0.001$) indicating that tissue uric acid concentrations are dependent on harvesting technique.

Vitamin E concentrations did not change significantly despite the notable lipid oxidation that occurred in the tissue and there were no differences between the rested and the exhausted groups (Figure 4.6).

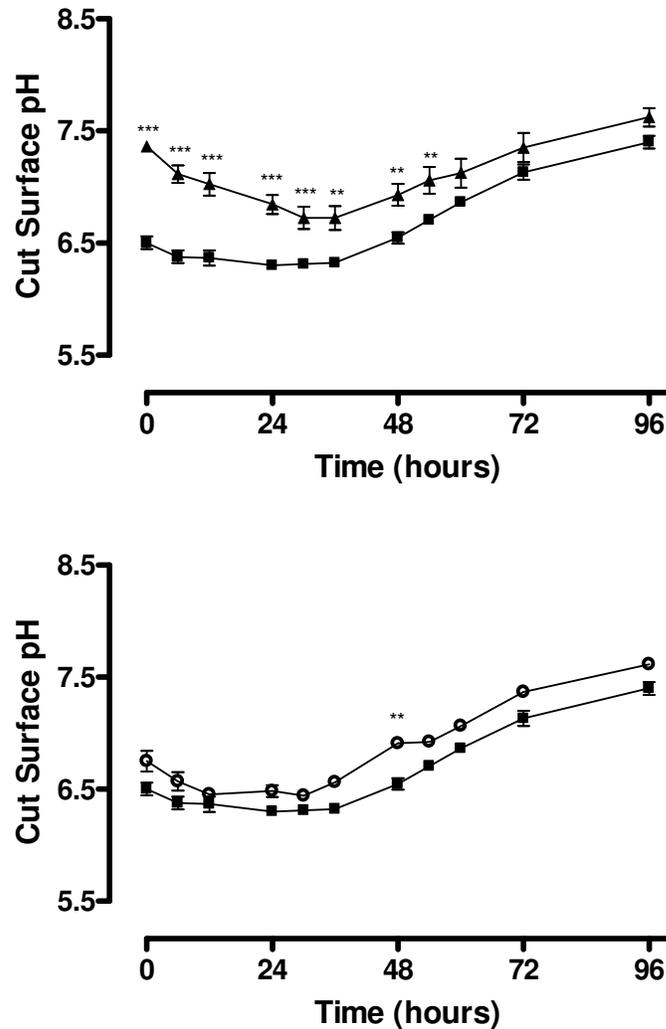


Figure 4.1 Mean cut surface pH (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle), exhausted (square) and exhausted fish harvested using rested harvesting techniques (open circle). Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data (p<0.001). Results from Bonferroni post-tests comparing treatments to standard exhausted harvesting are indicated (** p<0.01, *** p<0.001).

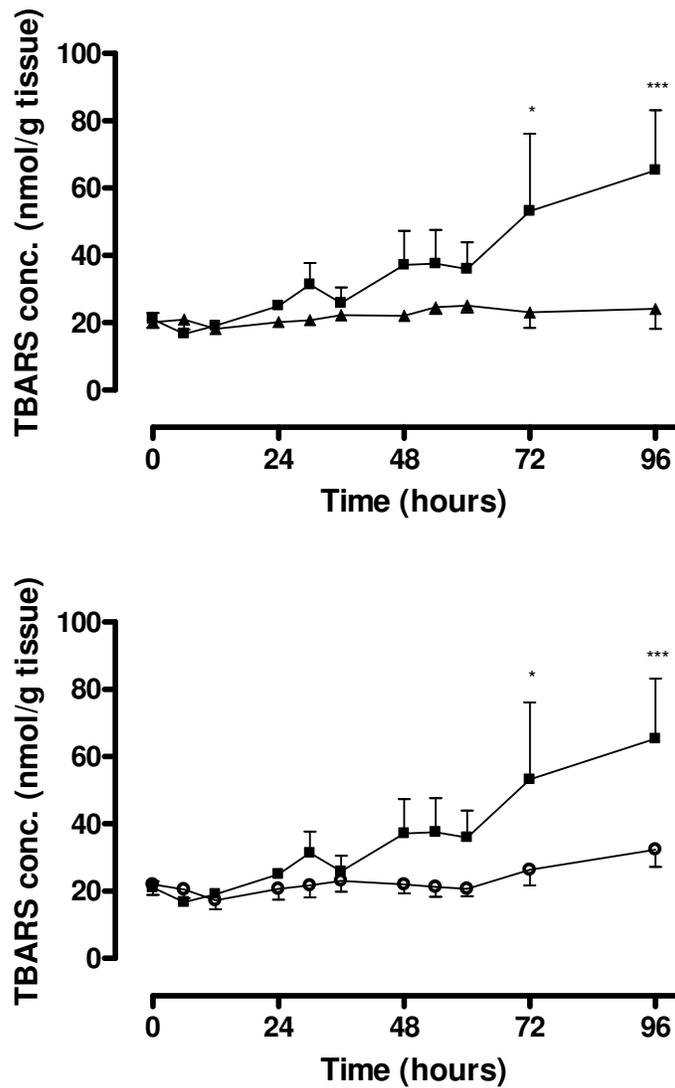


Figure 4.2 Mean TBARS concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle), exhausted (square) and exhausted fish harvested using rested harvesting techniques (open circle). Error bars indicate SEM. Two-way repeated measures ANOVA was performed on these data. Results from Bonferroni post-tests comparing treatments to standard exhausted harvesting are indicated (* p<0.05, *** p<0.001).

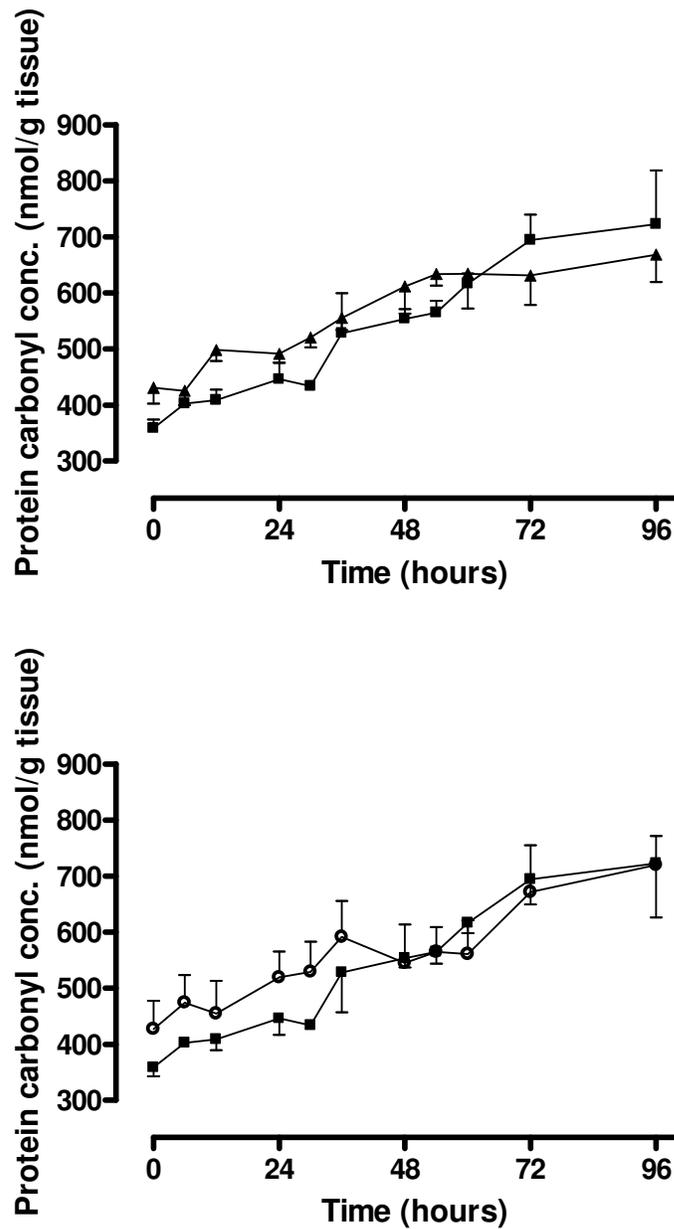


Figure 4.3 Mean protein carbonyl concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle), exhausted (square) and exhausted fish harvested using rested harvesting techniques (open circle). Error bars indicate SEM. Two-way repeated measures ANOVA showed no significant differences between treatments. Time was a significant source of variation ($p < 0.001$).

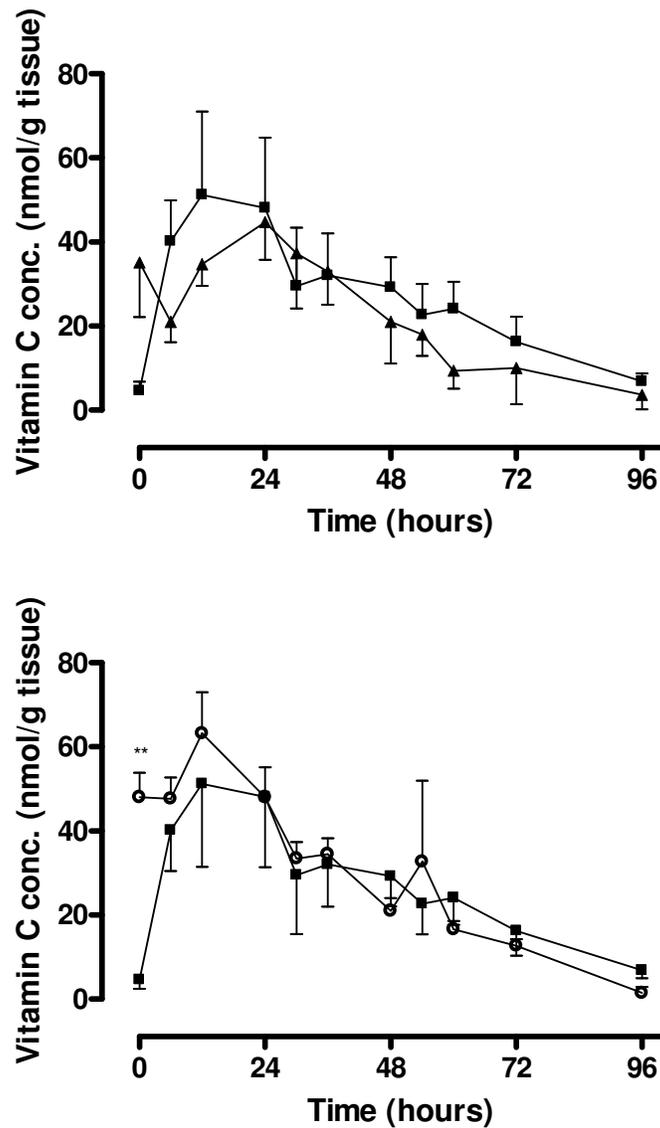


Figure 4.4 Mean vitamin C concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle), exhausted (square) and exhausted fish harvested using rested harvesting techniques (open circle). Error bars indicate SEM. Two-way repeated measures ANOVA was performed on these data. Results from Bonferroni post-tests comparing treatments to standard exhausted harvesting are indicated (** p<0.01).

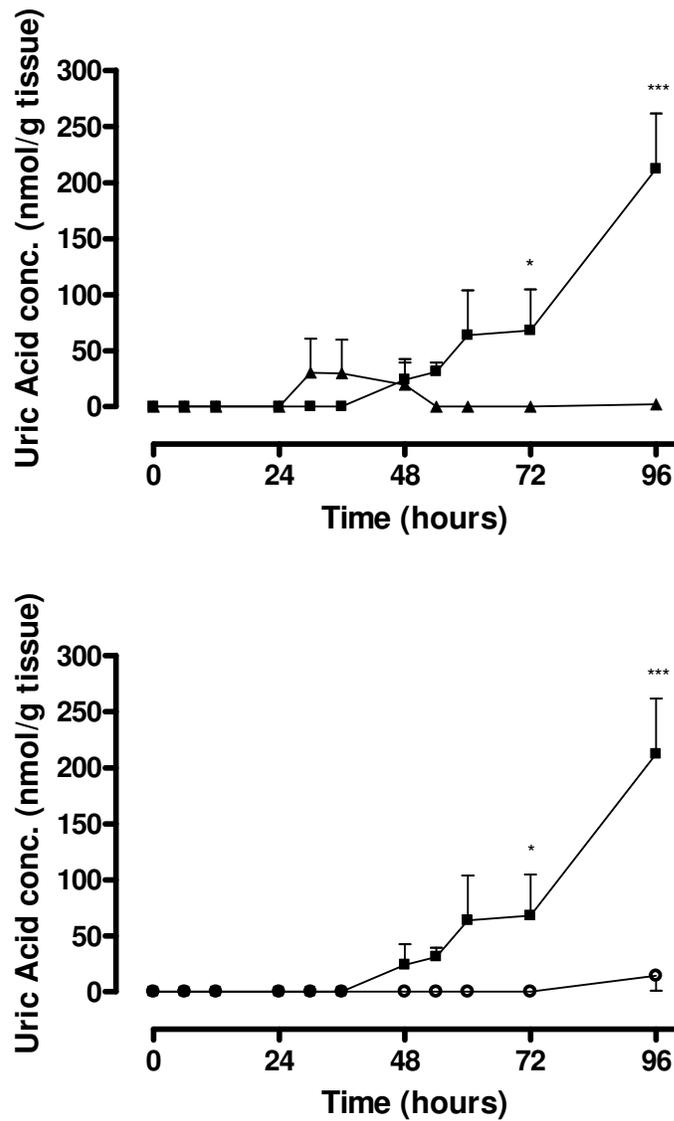


Figure 4.5 Mean uric acid concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle), exhausted (square) and exhausted fish harvested using rested harvesting techniques (open circle). Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.001$). Results from Bonferroni post-tests comparing treatments to standard exhausted harvesting are indicated (* $p < 0.05$, *** $p < 0.001$).

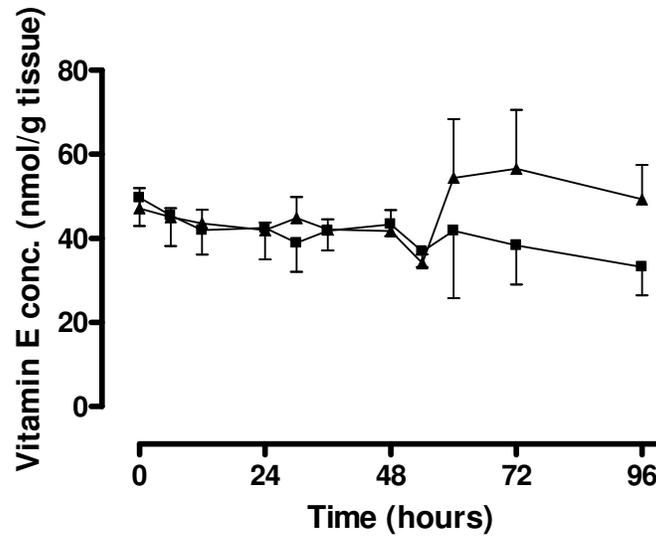


Figure 4.6 Mean vitamin E concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) salmon. Error bars indicate SEM. Two-way repeated measures ANOVA showed no significant differences between treatments or sources of variation.

4.3.2 Protein oxidation does not occur in tissue isolated from exposure to air

As the surface of the fillets was exposed to oxygen from the surrounding air, the role this played in the progression of oxidation was of interest. Because the tissue in the centre of the fillets was isolated from the air by the surrounding tissue, comparisons of the protein oxidation that occurred in tissue sampled from the fillet surface to that in the fillet centre allowed characterization of the role that air exposure plays in the progression of protein oxidation.

Unlike surface samples, samples from the centre of rested fillets did not show increased levels of protein oxidation (Figure 4.7). Two-way ANOVA showed that sampling location was a very significant source of variation ($p < 0.001$) and Bonferroni post-tests showed significantly higher levels of protein oxidation in surface samples at 36, 72 and 96 hours.

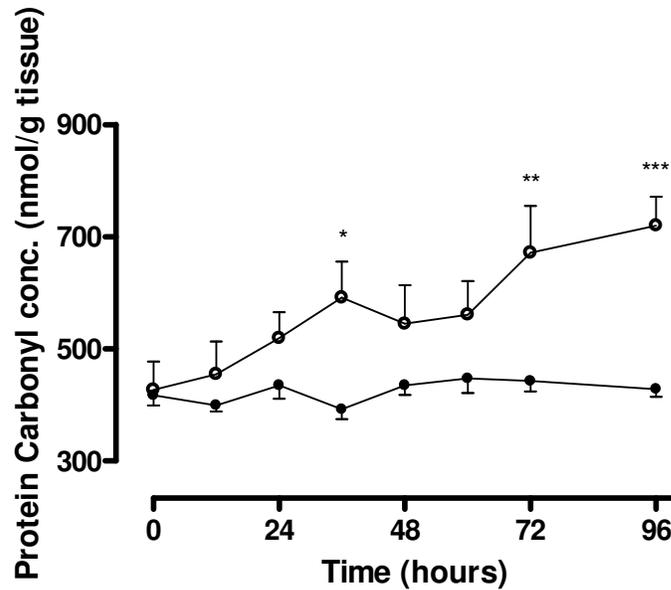


Figure 4.7 Mean protein carbonyl concentrations (n=4) from the surface (open circle) and centre (circle) from the white muscle of salmon tail fillets from exhausted fish harvested using rested harvesting techniques and stored at 15°C in air. Error bars indicate SEM. Two-way repeated measures ANOVA showed sampling location ($p < 0.001$) and time ($p < 0.05$) to be significant sources of variation. Results from Bonferroni post-tests comparing sampling locations are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.3 Tissue isoeugenol concentrations do not decrease in response to tissue oxidation

Determining the concentrations of isoeugenol in the fillets during storage allowed more specific inferences on its behaviour in the tissue to be made, in particular whether it was functioning as a chain breaking antioxidant. Isoeugenol concentrations from surface samples of white muscle increased consistently with time, approximately doubling over 96 hours (Figure 4.8). Linear regression showed this trend significantly deviated from 0 ($p < 0.001$) and this may be indicative of surface tissue desiccation and/or that isoeugenol extraction from the tissue prior to analysis was changing in response to tissue degradation. However, despite these uncertainties, there was no indication that isoeugenol was broken down by oxidative processes in the tissue during storage.

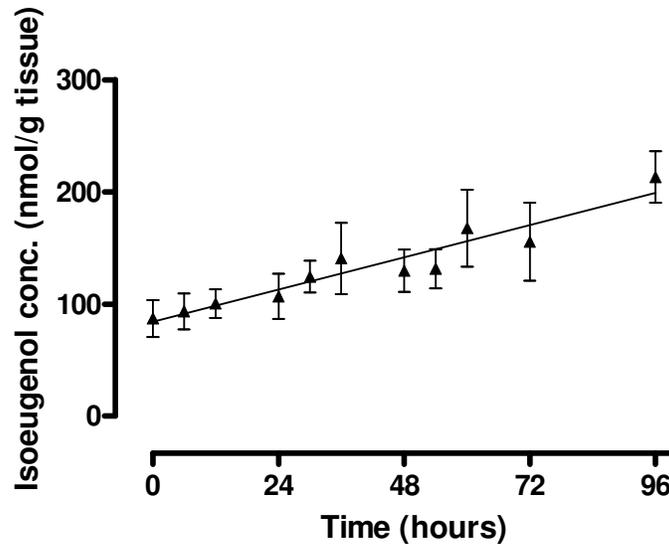


Figure 4.8 Mean isoeugenol concentrations from the white muscle of rested salmon tail fillets stored at 15°C for 96 hours. Linear regression showed that the slope deviated significantly from 0 (r^2 0.39, $p < 0.001$).

4.3.4 Oxidative processes and antioxidant loss in exhausted salmon tail fillets also occurs during storage at 6°C

The 15°C storage temperature used in this work was not representative of normal fish fillet storage practices. Therefore further experimental work was required at lower temperatures to ensure that the degradative processes we had measured at 15°C were not fundamentally altered by factors such as temperature related reaction rates and tissue gas permeabilities. Because the storage times for the two data sets differ, the data are not conducive to comparative statistical testing and therefore the data from the 15°C storage experiments are included in Figures 4.9 through 4.13 to allow visual comparison.

Cut surface pHs do not change significantly during 14 days storage at 6°C, although a rise is notable at the final measurement (Figure 4.9). The lower temperature limited TBARS formation over the first 4 days (Figure 4.10). However, rapid TBARS formation occurred between days 4 and 8 and the mean TBARS concentration of the tissue had significantly increased after 8 days storage at 6°C ($p < 0.01$). Protein carbonyls began to accumulate immediately during storage at 6°C (Figure 4.11) and had significantly increased after 6, 8 and 14 days ($p < 0.05$, 0.01 and 0.01 respectively). Vitamin C concentrations followed a similar trend to that seen during storage at 15°C, but the pre-storage mean was much higher and also the initial rise in concentrations was small (Figure 4.12). However, the high degree of variance means that Dunnett's post-tests did not show

any significant change from the pre-storage mean. Although uric acid did begin to accumulate after 8 days storage at 6°C, the change was not statistically significant (Figure 4.13).

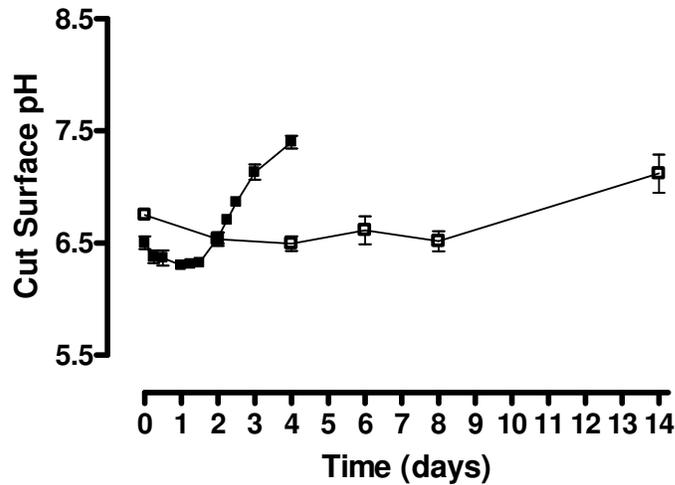


Figure 4.9 Mean cut surface pH (n=4) from the white muscle of salmon tail fillets stored at 15°C (squares) and 6°C (open squares) in air from exhausted fish. Error bars indicate SEM. One-way ANOVA was performed on the 6°C data and showed no significant differences.

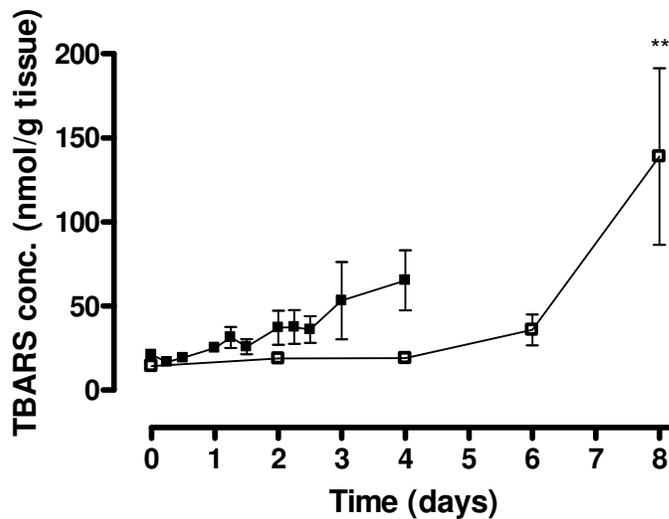


Figure 4.10 Mean TBARS concentration (n=4) from the white muscle of salmon tail fillets stored at 15°C (squares) and 6°C (open squares) in air from exhausted fish. Error bars indicate SEM. One-way ANOVA was performed on the 6°C data and showed significant differences between the means ($p < 0.01$). Results from Dunnett's post-tests comparing means to the pre-storage mean are indicated (** $p < 0.01$).

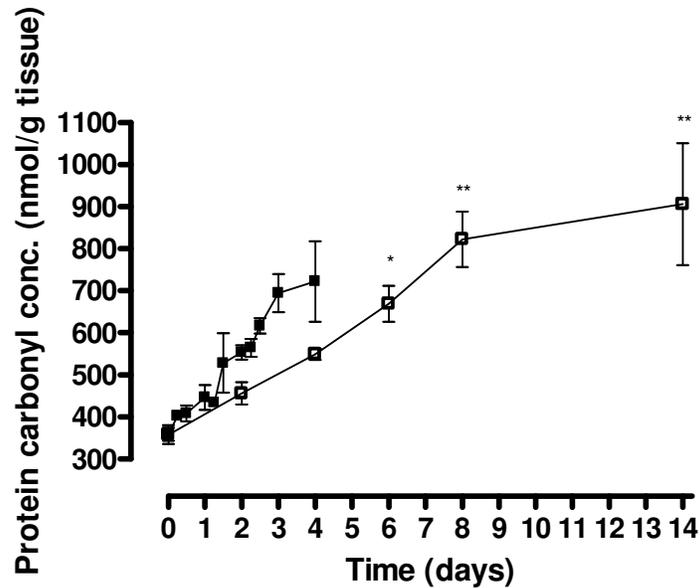


Figure 4.11 Mean protein carbonyl concentration (n=4) from the white muscle of salmon tail fillets stored at 15°C (squares) and 6°C (open squares) in air from exhausted fish. Error bars indicate SEM. One-way ANOVA was performed on the 6°C data and showed significant differences between the means ($p < 0.001$). Results from Dunnett's post-tests comparing means to the pre-storage mean are indicated (* $p < 0.05$, ** $p < 0.01$).

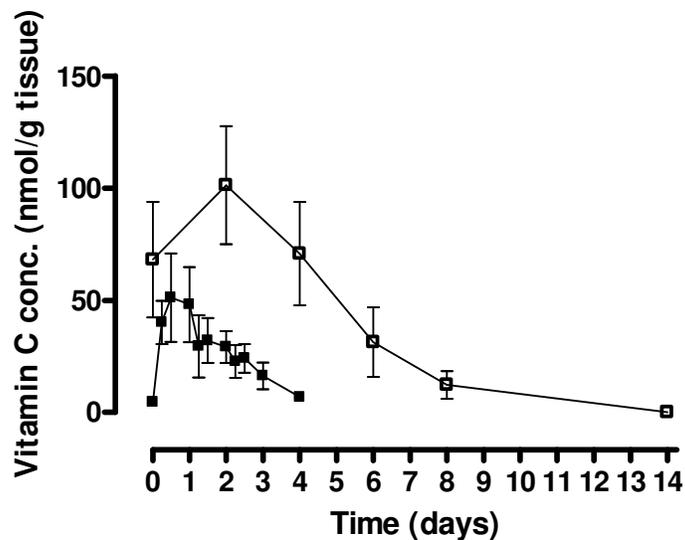


Figure 4.12 Mean vitamin C concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C (squares) and 6°C (open squares) in air from exhausted fish. Error bars indicate SEM. One-way ANOVA was performed on the 6°C data and showed significant differences between the means ($p < 0.05$). Results from Dunnett's post-tests comparing means to the pre-storage mean showed no significant differences.

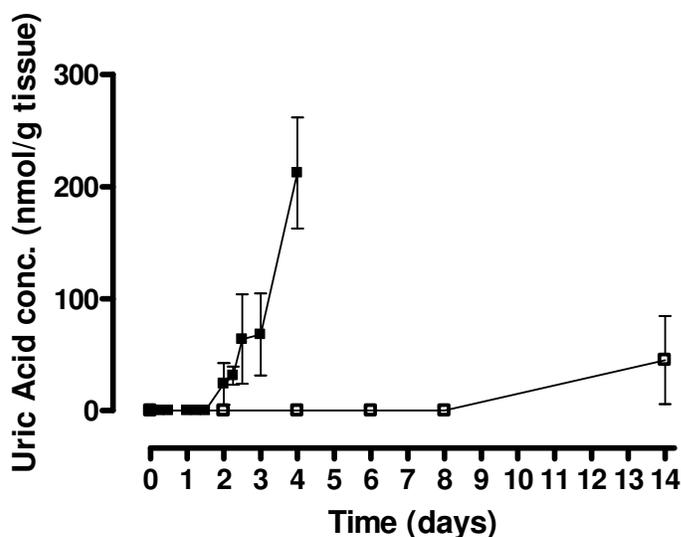


Figure 4.13 Mean uric acid concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C (squares) and 6°C (open squares) in air from exhausted fish. Error bars indicate SEM. One-way ANOVA was performed on the 6°C data and showed no significant differences.

4.3.5 Isoeugenol does not interfere with TBARS derivatization

As there was some risk that isoeugenol could interfere with TBARS derivatization, it was necessary to determine if this was an issue. Derivatization of the TBA-MDA adduct was not affected by the presence of 145 μM isoeugenol in solution. Linear regression of the two sets of standards showed r^2 values of 0.98 and 0.97 with and without isoeugenol respectively.

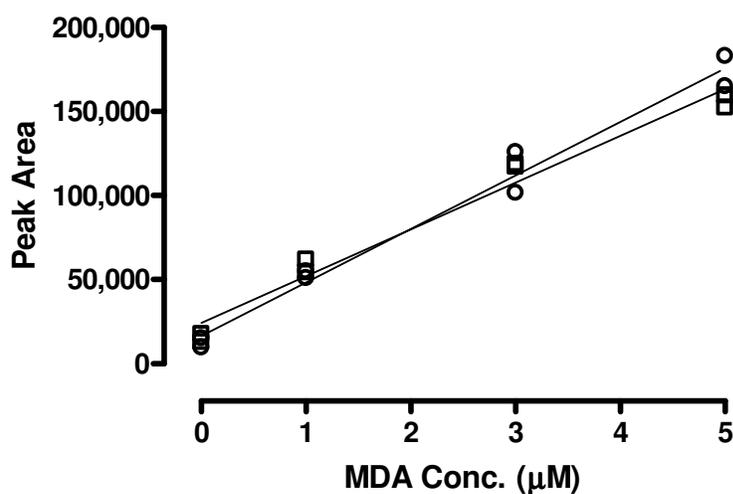


Figure 4.14 HPLC peak areas of known concentrations of MDA derivatized with TBA (square) and derivatized with TBA with 145 μM isoeugenol in solution (circle).

4.4 Discussion

The aim of this section was to provide profiles of the oxidation and loss of key antioxidants in chinook salmon white muscle as it transitioned from a viable to a non-viable state and then to the onset of autolysis and rancidity. Although there is no absolute time point at which these transitions take place, the complete loss of metabolic fuel within tissue stored at 15°C is shown in Chapter 5 to occur between 12 and 24 hours and this likely represents the loss of tissue viability. The inflection point in cut surface pH at 36 hours is a likely indicator of the onset of rancidity as it coincided with the development of a noticeable odour (Figure 4.1). These tissue states and the time points at which they occur are particularly relevant to the following discussion.

High or increasing cut surface pHs are indicative of fish products that are showing signs of rancidity (Bagni et al. 2007; Mugica et al. 2008). This work (Figure 4.1) shows cut surface pH profiles typical of both rested and exhausted white muscle (Black 2002; Bosworth et al. 2007). Rested tissue showed high cut surface pHs that declined slowly over 36 hours, while exhausted tissue showed low pHs that remained relatively stable over 36 hours. Particularly notable in these data is the time point at which pHs began to rise. In all three groups this happened around the 36 hour time point. This shows that despite the improved metabolic state of the tissue immediately post-harvest, the ensuing metabolic rundown does not delay or provide any protection from the onset of autolytic processes. Two main factors contributing to fillet spoilage are autolytic processes and microbial colonization (Aubourg et al. 2005; Aubourg et al. 2007; Mugica et al. 2008). Microbial colonization has been shown to occur relatively suddenly during storage (Undeland et al. 1999; Aubourg et al. 2007) and is likely it was a contributing factor to fillet properties in this work at the later time points and potentially played a role in the common cut surface pH inflection points at 36 hours. Measurements of microbial colonization were not possible without removing the fillets from the temperature controlled bins and therefore would have jeopardized the storage protocol. Also, this data may have shown aspects of tissue chemistry were generally related to microbial activity, but would not have provided specific details of the chemistry involved. However it is likely that microbial colonization was a contributing factor to tissue chemistry in this work.

Lipid oxidation has been shown to be an important factor in the spoilage of many seafood products and TBARS are often utilized as a means of measurement (Lee and Toledo 1977; Tichivangana and Morrissey 1982; Undeland et al. 1999; Undeland 2001;

Hamre et al. 2003). Figure 4.2 shows significant lipid oxidation occurred in exhausted fillets towards the end of the storage period. The use of AQUI-S™ during the harvesting procedures, regardless of the metabolic state of the fillet, eliminated the late stage lipid oxidation. This indicates that isoeugenol, the active ingredient of AQUI-S™, helps to stabilize the lipid fraction of the tissue and inhibit oxidation. This observation is limited to the lipid fraction however as no inhibition of protein oxidation was seen. The mechanism in which this takes place is not clear; however isoeugenol has a number of properties that may contribute. Firstly, both eugenol and isoeugenol have been shown to have antioxidant properties (Davcheva et al. 1995; Uchida et al. 1996) and stabilize oil in water emulsions (Cuvelier et al. 2003). There is also the possibility they may interact with endogenous antioxidants (Fujisawa and Kadoma 2006). Although, like a number of antioxidants, isoeugenol has also been shown to function as a prooxidant (Fujisawa et al. 2002; Atsumi et al. 2005) and therefore its contribution to redox chemistry can be considered to be dependent on its environment. Given that eugenol and isoeugenol are structural isomers and their radical scavenging ability is very similar, observations that isoeugenol is a more potent antioxidant than eugenol require explanation (Davcheva et al. 1995; Ito et al. 2005). Ito et al. (2005) suggested that the configuration of isoeugenol allows it to more strongly bind Fe^{2+} and maintain it in its reduced state. The prevention of Fe^{2+} , released predominantly from the myoglobin found in fish muscle (Baron and Andersen 2002; Chaijan et al. 2005), from contributing to the redox environment certainly has the potential to have a large influence on the susceptibility of stored seafood products to oxidation. Isoeugenol has also shown antimicrobial activity (Mansour et al. 1996), although the tissue concentrations found in this work may be too low for this to be a major contributing factor and work by Bosworth et al. (2007) showed no reduction in microbial counts on channel catfish fillets following rested harvesting.

The slow and continual rise of isoeugenol concentrations in rested fillets shown in Figure 4.8 may be characteristic of desiccation of the tissue surface, although the extraction procedure used in this work was simple and its effectiveness is unknown. Also, the purine nucleotide data displayed in Chapter 5 suggests that potential dessication of the surface tissue was not as great as indicated by the isoeugenol accumulation shown in Figure 4.8. Despite potential inaccuracies in the technique it remains very likely that it was adequate to show large changes and/or depletion of tissue isoeugenol concentrations. A stronger solvent to extract the lipid soluble compounds from the tissue may improve this technique and further work may be required to optimize it.

Although it has been reported that the tissue antioxidants vitamin C and vitamin E can reduce oxidized isoeugenol (Priyadarsini et al. 1999), the fact that tissue isoeugenol concentrations do not begin to decrease as tissue vitamin C concentrations approach zero and that rested harvesting does not alter tissue vitamin E loss rates indicates that there was little interaction in the fillet. This, and the fact that isoeugenol is not lost through oxidative processes, mean that inhibition of lipid oxidation in salmon fillets may be predominantly due to its ability to bind and maintain iron in its reduced state (Ito et al. 2005). As isoeugenol is lipid soluble it is also likely that the isoeugenol present in the fillets is associated with the lipid fraction of the tissue and, as shown in this work, would therefore not provide protection from oxidation for the protein fraction.

Measurement of protein oxidation in fish products has only begun relatively recently (Kjaersgard and Jessen 2004; Kjaersgard et al. 2006), but given the high protein content of fish muscle (Kiesling et al. 2004), protein oxidation is likely to be an important indicator of tissue oxidation. Measurements of protein oxidation are difficult due to the wide range of products produced and therefore the most frequently used techniques rely on general markers of protein oxidation such as protein carbonyls. Although increases in protein carbonyl concentration were seen in all of these fillets (Figure 4.3), desiccation may have been more pronounced than anticipated and therefore provide a confounding factor. However, the accumulation of protein oxidation in fish fillets during storage (Kjaersgard and Jessen 2004; Kjaersgard et al. 2006) and during stress in eelpouts (Heise et al. 2006) means that protein oxidation should not be overlooked and developments in analytical techniques should be monitored.

The lack of protein oxidation in samples taken from the centre of fillets (Figure 4.7) indicates that atmospheric oxygen and/or cellular disruption of the cut surface are playing primary roles in initiating oxidation on the surface of these fillets. High oxygen concentrations in stored meat products have been linked to oxidation (Bjerkeng and Johnsen 1995; Martinez et al. 2006). The processes initiating oxidation in the presence of oxygen are not certain but are likely to involve the Fenton reaction (Fe^{2+} reacting with H_2O_2 to produce alkoxy and peroxy radicals) and/or the Haber-Weiss reaction (Fe^{3+} reacts with O_2^- and forms a hydroxyl radical) (Halliwell and Gutteridge 1999). The isolation of tissue from air may limit the formation of H_2O_2 and O_2^- , significantly slowing tissue oxidation. Although limited measurements were taken in this work from internal tissues and desiccation may have been a confounding factor, it is likely that the removal of oxygen and/or iron from the tissue will reduce or eliminate oxidation. This is supported by the

successful application of modified atmosphere packaging to fish products (Fagan et al. 2004; Goulas and Kontominas 2007).

Measurements of tissue vitamin C concentrations (Figure 4.4) consistently showed high degrees of variance among the fillets. High degrees of variance have been shown in the blood plasma of some fish species (Gieseg et al. 2000) and, given the high degree of specificity and reproducibility of the assay procedure employed in this work (Levine et al. 1999), it is likely that this is reflective of the properties of the tissue and/or the population. Rapid changes in vitamin C were seen over the first 24 hours of storage, although the large SEMs prevented many of these differences from achieving statistical significance. The increase in concentrations seen between some time points was unexpected. However, vitamin C can be readily cycled between its oxidized and reduced states in skeletal muscle (Savini et al. 2005) and contributes not only to redox chemistry but is also important in a number of biological processes (Savini et al. 2005; Lall and Lewis-McCrea 2007). The assay used for this work does not detect dehydroascorbate. Given its wide ranging contribution to tissue chemistry the fluctuations in concentration as tissue viability is lost is perhaps indicative of its general importance. The time point at which concentrations stabilize possibly represents the point at which normal metabolic chemistry was no longer functional and vitamin C began functioning strictly as a redox influencing factor in the non-viable tissue. As the tissue ages, vitamin C is continually lost and approaches zero at the end of the storage period. Interestingly, there is no noticeable change in loss rate at time points where increased lipid oxidation begins. This provides further evidence that the lipid fraction and the water soluble fraction of non-viable tissues do not interact as much as they do in viable tissues.

Uric acid is the end point of purine metabolism and the buildup of its precursors hypoxanthine, inosine and inosine monophosphate (IMP) has been linked to metabolic status in transplant organs (Vigues et al. 1993; Domanski et al. 2007) and has also been used as a measure of freshness in stored fish (Ryder 1985; Luong et al. 1989; Luong and Male 1992; Carsol et al. 1997). Uric acid concentrations are displayed in Figure 4.5. Accumulation of uric acid began in exhausted fillets from 36 hours: a time point at which both cut surface pH and TBARS concentrations also began to rise. No associated rise was seen in either group of fillets harvested using AQUI-STM, indicating that anaesthesia and/or isoeugenol exposure has altered conditions in such a way that purine chemistry in the fillets was affected. As these changes were taking place at such a late stage in the tissue, following purine chemistry presented an opportunity to examine these changes upstream at

time points which may be relevant more specifically to cell death in fresh tissue. An HPLC assay allowing measurement of purine nucleotides, nucleosides and purine bases (Furst and Hallstrom 1992) was adapted for our equipment and utilized to look at the purine chemistry of rested and exhausted fillets. These data and detailed discussion is presented in Chapter 5 of this thesis.

The role of vitamin E *in vivo* is contentious. It has been shown to have both pro and antioxidant activity (Thomas et al. 1995; Kontush et al. 1996; Neuzil et al. 1997) and it has been suggested that it is involved in a number of cellular processes that are independent of its antioxidant function (Azzi et al. 2004; Zingg and Azzi 2004). Other researchers maintain that these processes are strictly a function of its antioxidant activity (Traber and Atkinson 2007). This has prompted comment by Brigelius-Flohe and Davies (2007) that there is a great deal of work left to be done to understand the functions of vitamin E. Despite the contentious nature of the literature, the results of this work were not difficult to interpret. Vitamin E concentrations (Figure 4.6) in fresh tissue were slightly lower, but comparable to other work (Johnston et al. 2006). There was a slight downward trend over the storage period, but the changes were not significant and the rate of loss did not change despite the rapid onset of lipid peroxidation. In these fillets it is therefore not possible to link tissue vitamin E concentrations to cell death. Also, although vitamin E concentrations have been linked to tissue stability (Schaefer et al. 1995; Jensen et al. 1998; Huang et al. 2004), any connection to tissue lipid peroxidation is not obvious from this work.

The majority of this work has been carried out at 15°C. This was to allow rapid profiling and assessment of the oxidative processes occurring in salmon muscle tissue and the analytical techniques that we had chosen to monitor these changes. Fillets stored at 15°C underwent the processes of cell death and the onset of rancidity well within the 96 hour experiment time, allowing us to generate a complete profile. 15°C was also below the upper temperature limit at which chinook salmon can be tank reared (Jerrett et al. 2000) and therefore cell viability loss in the fillets due to temperature stress was very unlikely. The data collected from fillets stored at 15°C indicated that the presence of AQUI-S™ in the fillets was inhibiting lipid oxidation, but care was required before this could be extrapolated to other storage temperatures. Therefore it was necessary to investigate whether the white muscle of salmon fillets would undergo the same chemical processes in a classical temperature dependent manner, or whether temperature was having a much more fundamental impact on the chemical processes occurring in post-harvest tissue. We

decided to use exhausted fillets stored at 6°C (the lowest maintainable temperature that could be achieved by our equipment) as that would allow us to monitor TBARS, which had not increased in rested fillets. Fillets stored for up to 2 days at 15°C were generally comparable to fillets stored for up to 4 days at 6°C. Higher cut surface pHs (Figure 4.9) from the fillets stored at 15°C were possibly a result of increased microbial growth at a more favorable temperature. Interestingly TBARS concentrations (Figure 4.10) took longer to begin to rise, but then rose quickly at 6°C. This may show that despite inhibiting oxidation during early storage, cold temperatures may stabilize the products of lipid oxidation when they do begin to form. Protein oxidation rates (Figure 4.11) were generally comparable and although vitamin C concentrations (Figure 4.12) were higher in the fillets stored at 6°C, the trend in concentrations over time was similar. The differences in vitamin C concentrations may be related to seasonal variation, as the fish were sampled at different times of the year, and/or size differences between the respective groups. Uric acid concentrations (Figure 4.13), despite rising, did not change significantly during storage at 6°C. Although these data do show temperature dependent variations in the trends and therefore that some care is required when making extrapolations across storage temperatures, they do show that the factors measured in this work are not fundamentally altered by temperature and that tissue chemistry during storage at 15°C is likely to be similar to that at other storage temperatures. Further work would be required to confirm this, particularly for frozen storage.

Although it seemed very unlikely, and there was no obvious mechanism through which isoeugenol could affect the TBARS assay, it seemed prudent to check. Isoeugenol added to MDA standards at approximately twice the concentration found in anaesthetized fish tissue showed no signs of interference.

4.4.1 Summary

There were clear differences in the tissue chemistry of the white muscle from rested and exhausted chinook salmon. Some of these properties, such as reduced lipid oxidation and altered purine chemistry, appear to be conferred not by the physiological state of the tissue during harvest, but by the presence of isoeugenol in the tissue. This may be mediated by the ability of isoeugenol to maintain iron in its reduced state. The ability of isoeugenol to inhibit lipid oxidation in salmon fillets is notable. However, as lipid oxidation occurs

relatively late in the tissue degradation process this may not be relevant to commercial processes.

The techniques used in this work do not indicate clear relationships between oxidative damage and the loss of tissue antioxidants with cell death. More sensitive techniques to measure intracellular redox potential leading up to and during cell death may be necessary to advance our understanding in this area.

5 - ATP related compound profiles from the white muscle of rested and exhausted chinook salmon tail fillets during storage

5.1 Introduction

The aim of this chapter was to measure and follow the progression of ATP related compounds that result in uric acid accumulation in stored chinook salmon fillets. In Chapter 4, there was significantly reduced uric acid accumulation in rested fillets during storage at 15°C than in exhausted fillets. These changes occurred relatively late in the storage period however and were therefore not useful for making inferences on post harvest metabolic state or associated quality factors in the respective tissues. The similarity in structure of these compounds lends them to separation and quantification with HPLC. In this chapter we have utilized and modified an ion-pairing HPLC technique that allows simultaneous determination of these compounds (Furst and Hallstrom 1992), many of which are directly indicative of metabolic state and are also used as biochemical indicators of quality in seafood products (Ryder 1985; Vazquez-Ortiz et al. 1997; Dondero et al. 2004; Aubourg et al. 2007; Sallam 2007). In this chapter, a detailed picture of the effects of rested harvesting on post harvest metabolism and cell death and associated biochemical measures of quality from chinook salmon white muscle during storage at 15°C is presented.

5.2 Materials and Methods

Detailed methodology for this section can be found in Chapter 2. Two groups of chinook salmon were used to determine profiles of creatine compounds, nucleoside, nucleotides and related bases in rested and exhausted white muscle. The first group (n=4) was harvested using rested harvesting techniques and had a mean weight of 1754 ± 167 g. The second group (n=4) was exhausted by chasing during a simulated harvest and had a mean weight of 1735 ± 132 g. This was necessary as no harvesting was taking place at Isaac's salmon farm and was carried out with animal ethics approval from the Animal Ethics Committee (approval number 2006/24R). The fish were exposed to surrounding activity for a period of 2-3 minutes. A net was then placed in the tank and moved in a circular

motion for 60-90 seconds until locomotion was obviously impaired. The fish were then removed from the tank with the net, killed by cranial impact, bled and transported on ice to the University of Canterbury for fillet preparation.

Fillets were prepared from the left side of the fish beginning at the dorsal fin and running to the tail. The cut surface pH of the fillet was taken as the mean of six concurrent measurements from the D block, three each side of the spine beginning 10mm from the anterior end of the fillet. Samples were taken from the D block and freeze clamped and stored at -80°C for extraction. The fillets were placed in a temperature controlled bin at $15^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Repeated pH measurements and samples from undisturbed locations not including the posterior third of the fillet were taken at 0, 3, 6, 12, 24, 30 and 36 hour time points.

Tissue was sampled from both the exposed tissue and the unexposed tissue from the centre of the fillet after 30 hours storage. This was to determine if any differences, particularly in ATP concentrations, were present in tissue that was not exposed to air.

Samples were ground to a fine powder under liquid nitrogen, weighed and homogenized in 1.5ml of ice cold 0.4M PCA. The blade was rinsed in a small volume of PCA, pooled with the sample and the volume made up to 1.8 ml. This was centrifuged at 500 rpm for 5 minutes. 500 μl of the supernatant was removed, neutralized to pH 7.0 with 55 μl of 2 M K_2CO_3 and stored at -80°C for analysis.

Creatine compounds, nucleosides, nucleotides and related bases were separated by ion-pair liquid chromatography and quantified by UV-Vis detection. This technique was modified from the one used by Furst and Hallstrom (1992). The PCA extracted and neutralized muscle tissue samples were thawed and centrifuged at 10 000rpm for 5 minutes and 20 μl of the supernatant was injected onto the HPLC. Compounds were quantified by comparison to standard solutions of known concentration. K-values were calculated with the formula below as used by Saito (1959) and Aubourg (2007).

$$\text{K-value (\%)} = \frac{[\text{Ino}] + [\text{Hypoxanthine}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}] + [\text{IMP}] + [\text{Ino}] + [\text{Hypoxanthine}]} \times 100$$

Glutathione was derivatized with the fluorescent dye monobromobimane (MBB), separated by HPLC and quantified with fluorescence detection (Cotgreave and Moldeus 1986).

5.3 Results

The purpose of collecting cut surface pHs was to ensure the data in Chapters 4 and 5 were comparable, and also to correlate this measurement with concentrations of ATP related compounds in the respective tissues. The profiles were again typical of rested and exhausted tissues. The rested tissue showed significantly higher cut surface pHs from 0 through to 12 hours ($p < 0.001$). Of particular note, higher pH values were obtained from freshly prepared rested fillets than was seen in Chapter 4. This is likely to be a result of improved anaesthesia and animal handling techniques as discussed in Chapter 2. Also, the extra time point at 3 hours indicates a sudden acidification in fresh rested fillets followed by a period of stability through to 6 hours. This suggests that the metabolic rundown of the tissue takes place in a stepwise manner as opposed to a continuous rundown and that the sampling time points used in this work may create some artificial smoothing of the data.

The chromatograms showing the separation of creatine compounds, nucleosides, nucleotides and other bases from a rested and an exhausted salmon (Figures 5.2 and 5.3) clearly show the effectiveness of this HPLC technique and its suitability for use with fish tissue extracts. The depletion of ATP and creatine phosphate concentrations and the increase in IMP concentrations in the exhausted tissue is notable.

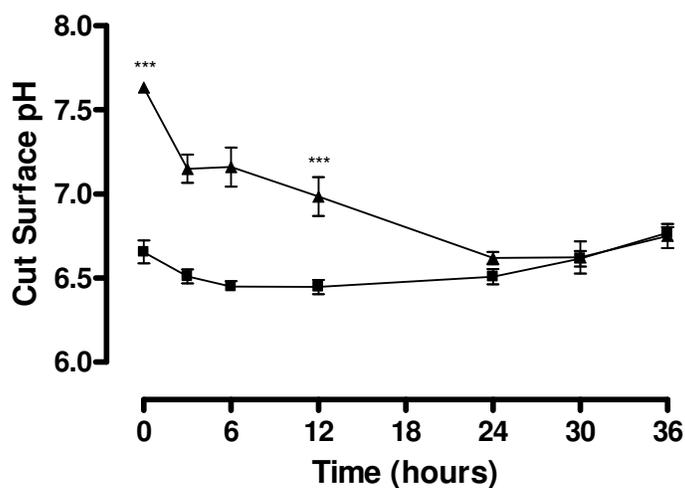


Figure 5.1 Mean cut surface pH ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.001$). Results from Bonferroni post-tests comparing treatments are indicated (***) ($p < 0.001$ inclusive of time points between those marked).

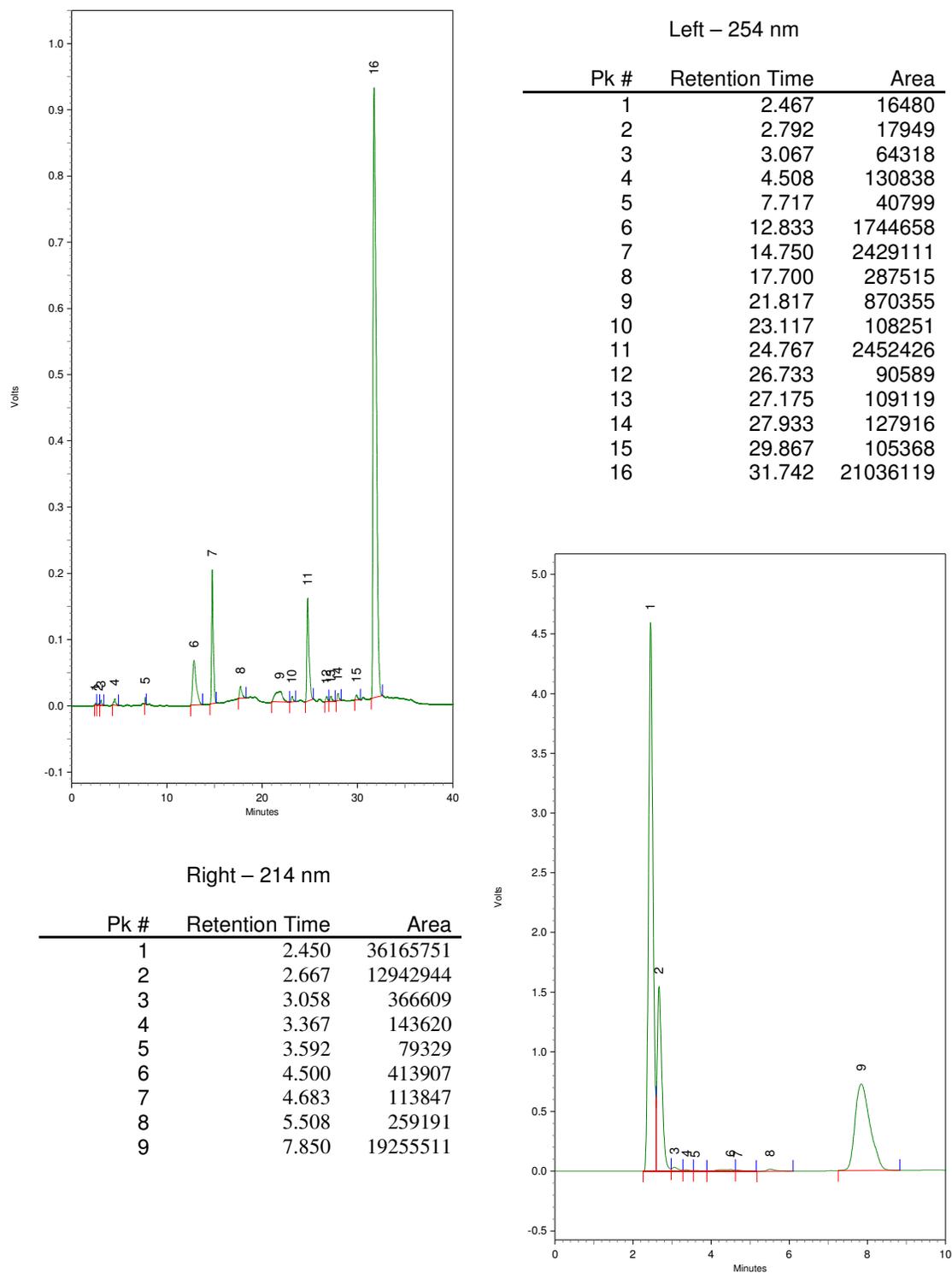


Figure 5.2 Simultaneously captured chromatograms at 254 and 214 nm from the extracted white muscle tissue of a chinook salmon following rested harvesting.

Peaks at 254 nm (top left): 4 = hypoxanthine, 6 = IMP, 7 = NAD⁺, 8 = AMP, 11 = ADP, 16 = ATP. Peaks at 214 nm (bottom right): 1 = creatine, 2 = creatinine, 9 = creatine phosphate.

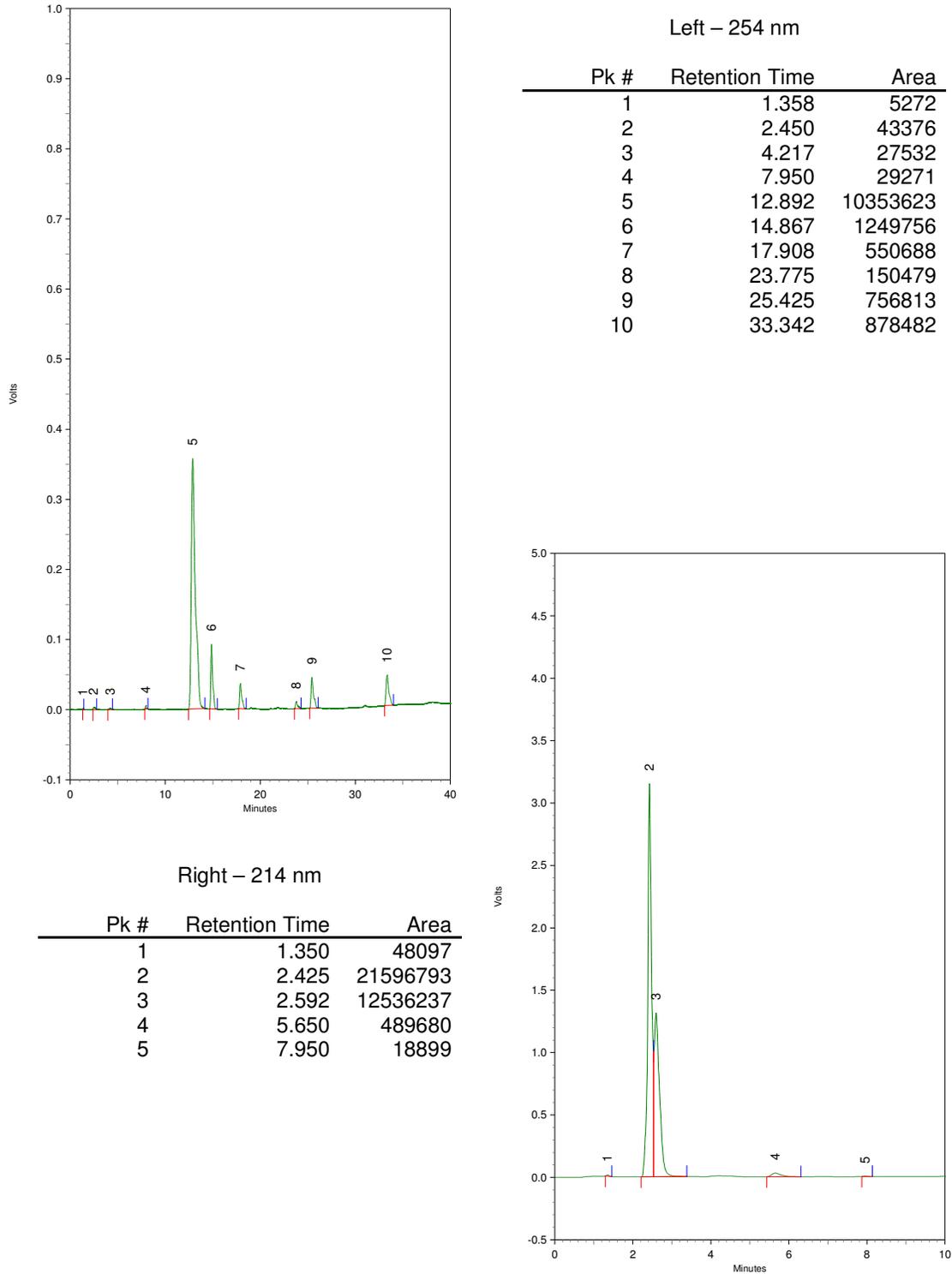


Figure 5.3 Simultaneously captured chromatograms at 254 and 214 nm from the extracted white muscle tissue of a chinook salmon following exhaustive harvesting. Peaks at 254 nm (top left): 3 = hypoxanthine, 5 = IMP, 6 = NAD⁺, 7 = AMP, 9 = ADP, 10 = ATP. Peaks at 214 nm (bottom right): 2 = creatine, 3 = creatinine.

5.3.1 Creatinine, creatine and creatine phosphate profiles

As creatine metabolism plays an important role in the metabolic function of skeletal muscle cells, the relative concentrations of creatine, creatine phosphate and creatinine and their relationship to cell death are of interest. Creatinine concentrations (Figure 5.4) in rested fillets at 0 and 3 hours were significantly lower than in exhausted fillets. There was a rapid rise over the first three hours of storage in rested fillets followed by a continuous rise through to 36 hours. No changes were seen over the first 6 hours of storage in exhausted fillets indicating extra pooling of creatinine in exhausted tissues.

Although some statistical differences existed in creatine concentrations (Figure 5.5) between rested and exhausted fillets the trend in both groups was similar, particularly over the first 6 hours during metabolic rundown in the tissue. Dehydration of the surface tissue may be playing a role in generating the steady increases in concentration of both creatinine and creatine over the 36 hour storage period.

Creatine phosphate concentrations (Figure 5.6) in fresh rested tissue were approximately $8.3 \mu\text{mol g}^{-1}$. This reduced rapidly over the first 3 hours of storage, then continued to reduce at a slightly slower rate and was depleted after 12 hours of storage. No creatine phosphate was detectable in exhausted tissue.

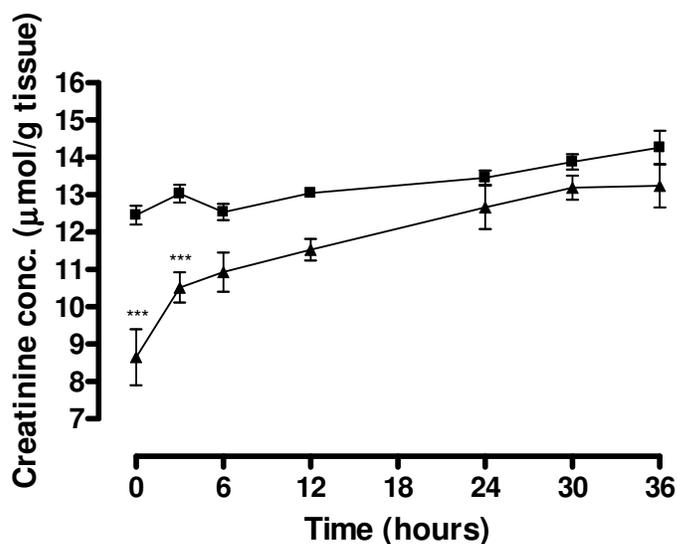


Figure 5.4 Mean creatinine concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (***) $p<0.001$ inclusive of time points between those marked).

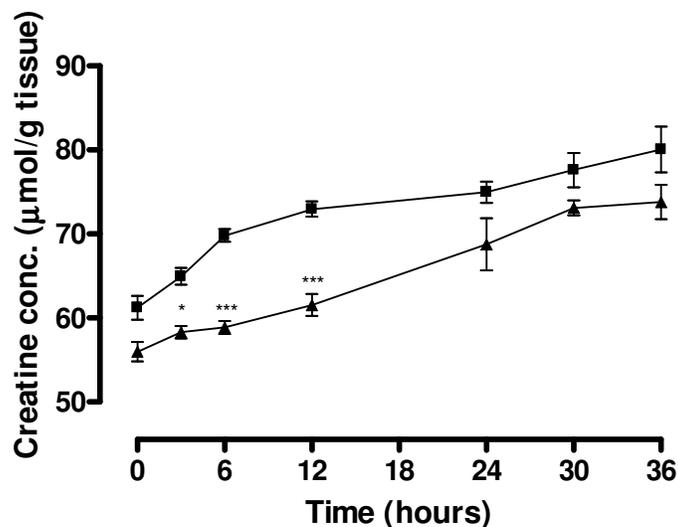


Figure 5.5 Mean creatine concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed time ($p<0.001$) and harvest type ($p<0.01$) as significant sources of variation. Results from Bonferroni post-tests comparing treatments are indicated (* $p<0.05$, *** $p<0.001$).

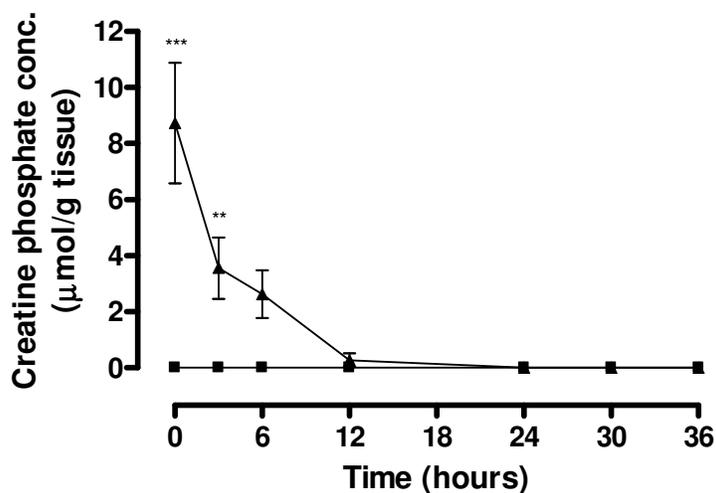


Figure 5.6 Mean creatine phosphate concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (** $p<0.01$, *** $p<0.001$).

5.3.2 Uric acid, hypoxanthine, inosine and IMP profiles

Measurements of uric acid, hypoxanthine, inosine and IMP are important because the concentrations of these compounds are indicative of the quality of fish tissues and they may also contribute to oxidative processes. The very high uric acid concentrations (Figure 5.7) that began to accumulate immediately in the tissue contrasted with the lower values shown in Chapter 4. Uric acid concentrations increased consistently in both groups. However, there was a 6 hour lag phase before uric acid concentrations began to increase in the rested group. After 6 hours the trends were very similar but the significant differences in concentrations that the lag phase in the rested group created remained throughout the storage period. These differences were highlighted by the significant interaction between the two data sets ($p < 0.001$).

Hypoxanthine concentrations (Figure 5.8) increased at very similar rates in both groups. The trends diverged after 24 hours storage and the rested group showed significantly higher hypoxanthine concentrations at 30 and 36 hours.

Inosine concentrations (Figure 5.9) rose and also showed a slight lag phase in the rested group. However the differences between the two groups were very small and none were statistically significant.

The trends in IMP concentrations (Figure 5.10) were very different in these two groups. IMP concentrations were significantly lower in the rested group in fresh tissue and increased rapidly over the first 3 hours, then more slowly and peaked after 12 hours storage before beginning a steady decrease. Exhausted fillets showed high IMP concentrations in fresh tissue that steadily decreased during 36 hours storage. These two data sets also showed significant interaction ($p < 0.001$).

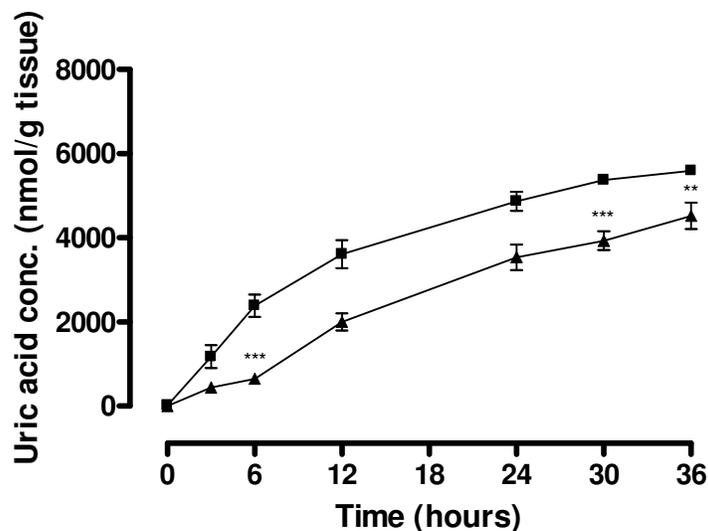


Figure 5.7 Mean uric acid concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (** $p<0.01$, *** $p<0.001$ inclusive of time points between those marked).

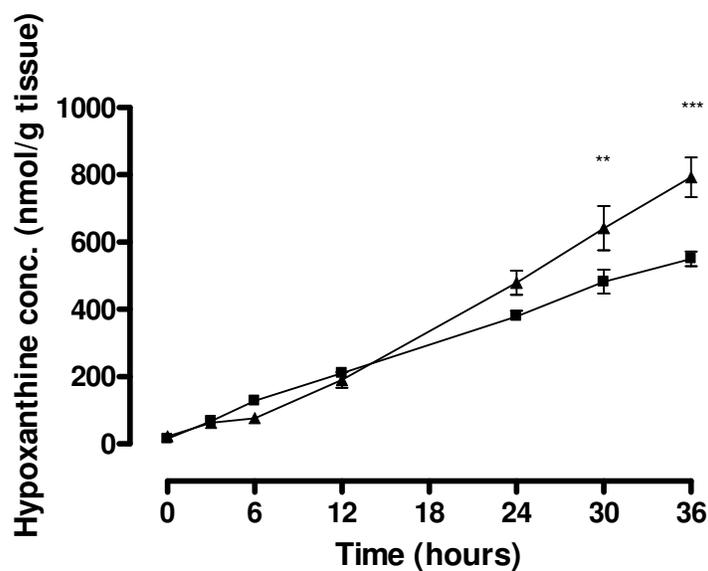


Figure 5.8 Mean hypoxanthine concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (** $p<0.01$ *** $p<0.001$).

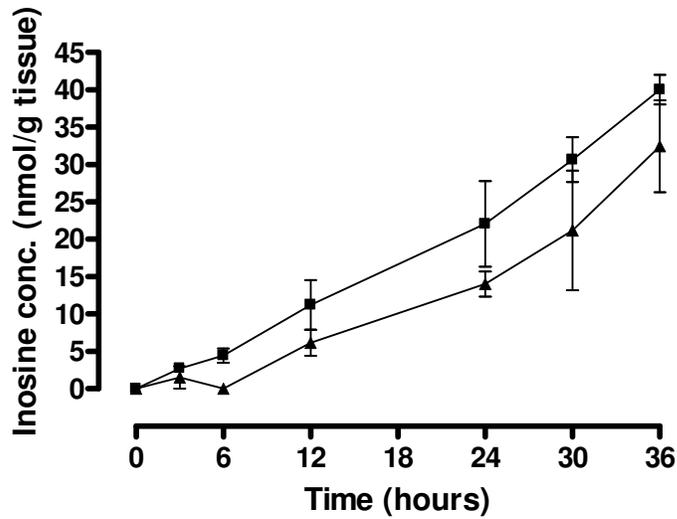


Figure 5.9 Mean inosine concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed time as a significant source of variation in these data ($p<0.001$).

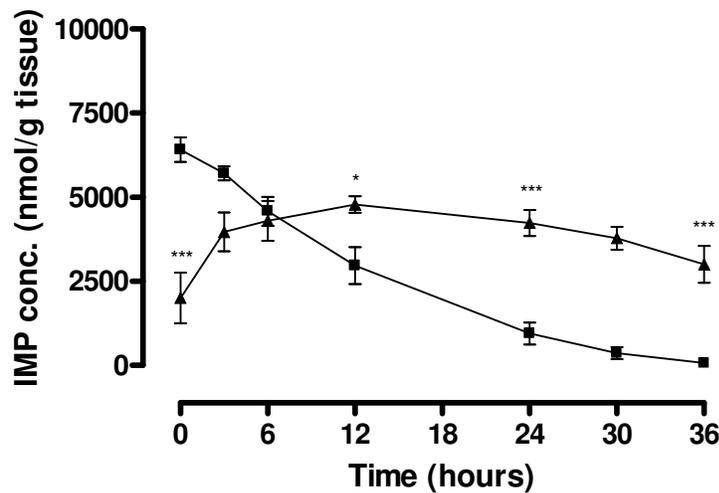


Figure 5.10 Mean IMP concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (* $p<0.05$ *** $p<0.001$ inclusive of time points between those marked).

5.3.3 NAD⁺, AMP, ADP and ATP profiles

NAD⁺, AMP, ADP and ATP are vital for both aerobic and anaerobic energy generation. Therefore the way in which their concentrations change leading up to and during cell death may advance understanding of this process and ultimately allow development of technologies that extend tissue viability post-harvest. Tissue NAD⁺ concentrations (Figure 5.11) in rested fillets show a rapid drop over the first 3 hours of storage followed by 3 hours of stable concentrations and a more gradual decline reaching near depletion after 24 hours. The period of stability is not present in exhausted fillets. They show an immediate and rapid decline in NAD⁺ concentrations and reach near depletion after only 12 hours. There was significant interaction between these data sets ($p < 0.001$).

AMP concentrations (Figure 5.12) in the two groups were significantly different in fresh tissue. Rested tissue had significantly lower concentrations that increased over the first 6 hours of storage. Exhausted tissue had higher AMP concentrations that reduced over the first 6 hours of storage. However both groups stabilized after 6 hours and no further changes were seen. The difference in trends seen in the first 6 hours of storage produced a significant interaction between these data sets ($p < 0.01$).

ADP concentrations (Figure 5.13) in rested tissue were stable for 6 hours before beginning to decrease and reaching their lowest concentration after 24 hours storage. The period of stability was only 3 hours in exhausted tissue and the decrease was very rapid, reaching its lowest point after just 6 hours storage. Again there was a significant interaction between these data sets ($p < 0.001$).

Tissue ATP concentrations (Figure 5.14) show very similar trends to both creatine phosphate concentrations and cut surface pH. Rested tissue shows high ATP concentrations that reduce rapidly during the first 3 hours of storage. The rate of decline reduces slightly after 3 hours and near depletion is reached after 24 hours. Exhausted tissue showed very low concentrations of ATP and near depletion occurred after 6 hours storage. These data sets also had significant interaction ($p < 0.001$).

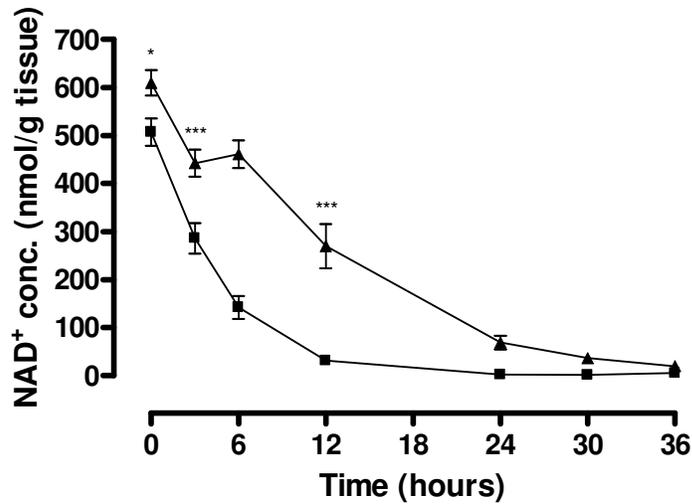


Figure 5.11 Mean NAD⁺ concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data (p<0.001). Results from Bonferroni post-tests comparing treatments are indicated (* p<0.05, *** p<0.001 inclusive of time points between those marked).

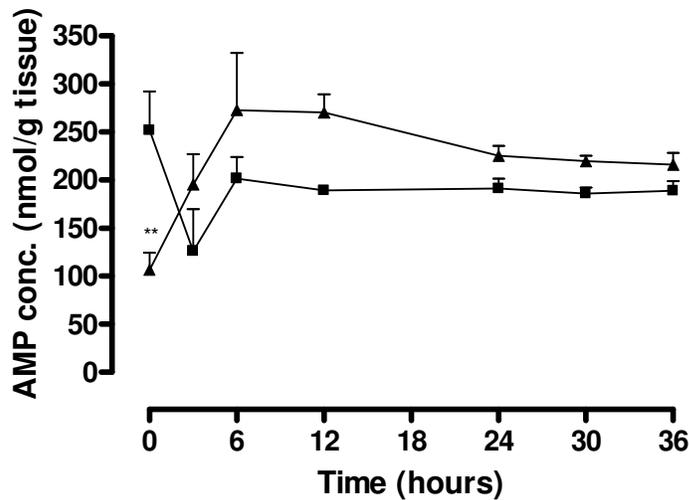


Figure 5.12 Mean AMP concentrations (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data (p<0.01). Results from Bonferroni post-tests comparing treatments are indicated (** p<0.01).

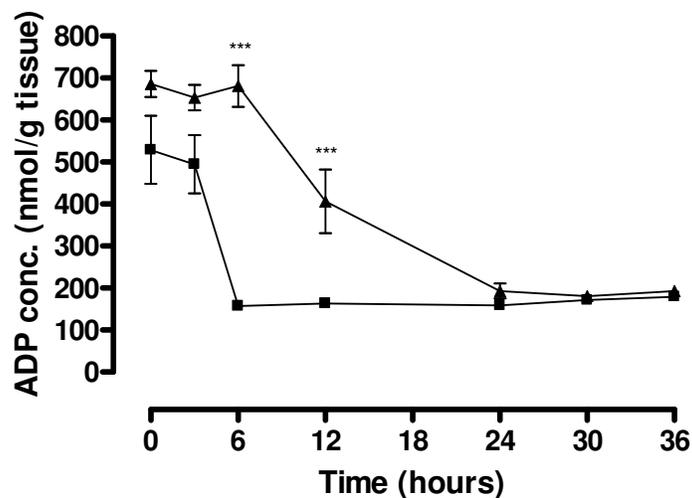


Figure 5.13 Mean ADP concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (***) ($p<0.001$).

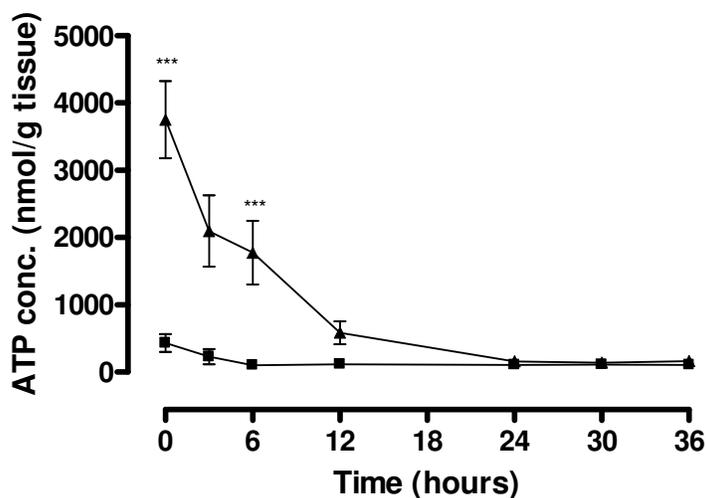


Figure 5.14 Mean ATP concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p<0.001$). Results from Bonferroni post-tests comparing treatments are indicated (***) ($p<0.001$ inclusive of time points between those marked).

5.3.4 ATP/ADP ratios, ATP/AMP ratios, ATP/IMP ratios and K-values

The ATP/ADP and ATP/AMP ratios (Figures 5.15 and 5.16) are indicative of the metabolic state of the tissue. Rested tissue shows a clear rundown in both of these ratios over 24 hours, whereas exhausted tissue shows almost no change in ratio during the storage period. There was significant interaction between the exhausted and rested groups in both these data sets ($p < 0.001$).

The ATP/IMP ratio (Figure 5.17) is also an indicator of metabolic state. In rested tissue it reduces very suddenly in the first 3 hours of storage and then more slowly, reaching a low point after 12 hours. In exhausted tissue the ATP/IMP ratio is very low in fresh tissue and remains low before starting to climb after 24 hours storage. There was also significant interaction between these data sets ($p < 0.01$).

K-values (Figure 5.18) are more indicative of the freshness of the tissue than the metabolic state. However, after 12 hours storage the K-values in exhausted tissue began to increase at a much faster rate than in rested tissue. There was significant interaction ($p < 0.001$) between these data sets.

The total tissue nucleotide pools (Figure 5.19) changed slightly during storage but remained comparable to concentrations in fresh tissue for the entire storage period. This indicates that many of these compounds remain in this chain of ATP related compounds for the entire storage period.

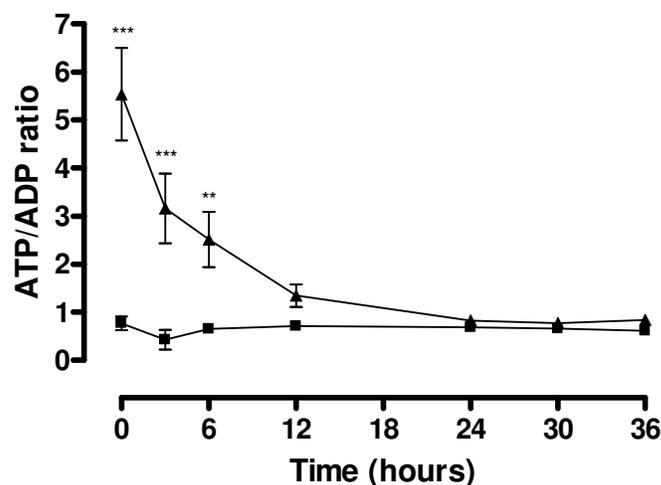


Figure 5.15 Mean ATP/ADP ratios ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.001$). Results from Bonferroni post-tests comparing treatments are indicated (** $p < 0.01$, *** $p < 0.001$).

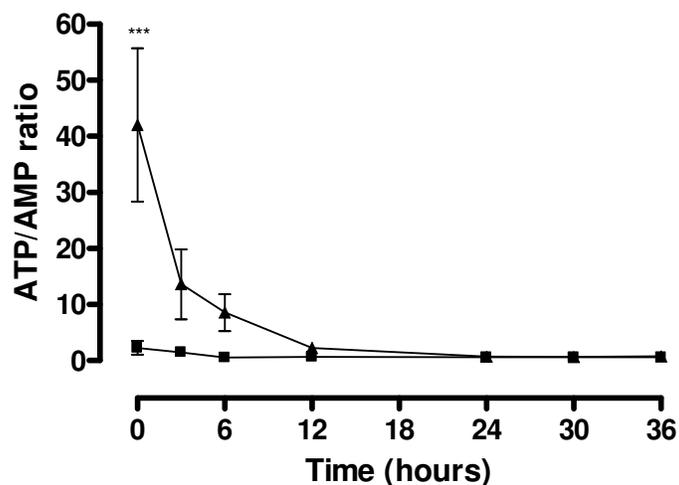


Figure 5.16 Mean ATP/AMP ratios (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.001$). Results from Bonferroni post-tests comparing treatments are indicated (***) $p < 0.001$).

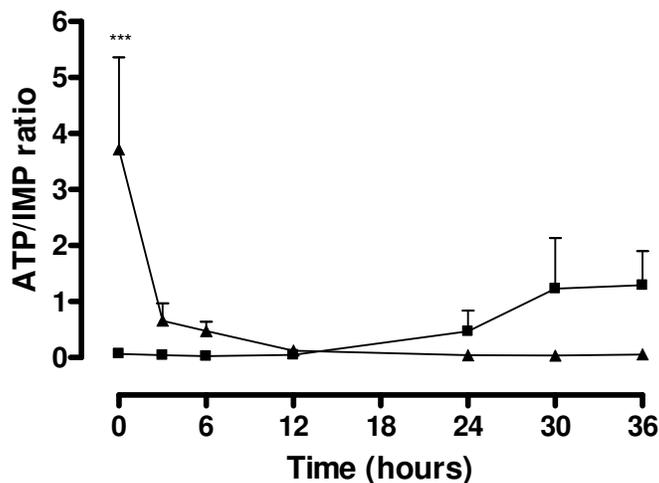


Figure 5.17 Mean ATP/IMP ratio (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.01$). Results from Bonferroni post-tests comparing treatments are indicated (***) $p < 0.001$).

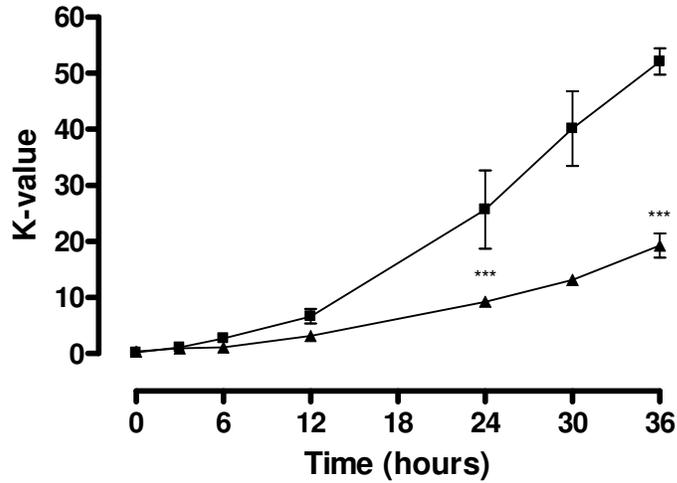


Figure 5.18 Mean K-values (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM. Two-way repeated measures ANOVA showed significant interaction in these data ($p < 0.01$). Results from Bonferroni post-tests comparing treatments are indicated (***) $p < 0.001$ inclusive of time points between those marked).

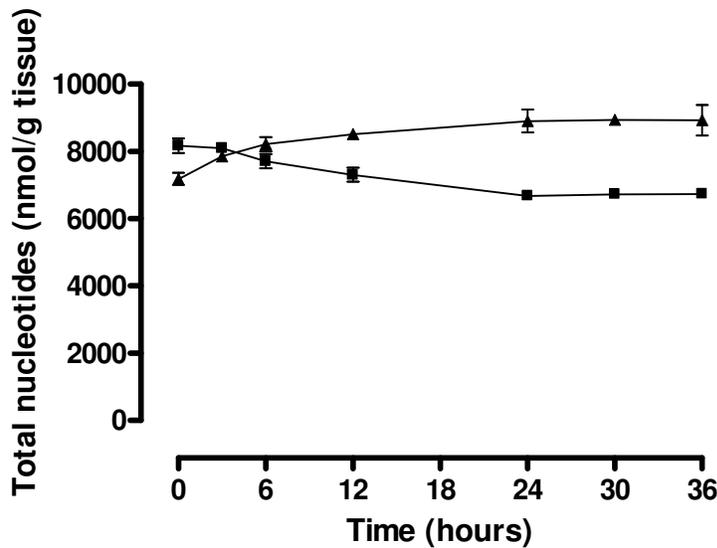


Figure 5.19 Mean total nucleotide pool (n=4) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM.

5.3.5 The predictive power of cut surface pH

Cut surface pH has been shown to be a useful indicator of metabolic state in freshly harvested fish (Black 2002; Janssen 2003). Of the 15 metabolite concentrations and ratios measured in this chapter, only IMP and AMP were not significantly correlated with cut surface pH. The predictive power of these correlations was variable with r^2 values ranging from 0.1 to 0.67. However, there were highly significant correlations ($p < 0.0001$) and relatively high r^2 values relating cut surface pH and the metabolites creatine phosphate, NAD^+ , ADP, ATP and the ATP/ADP ratios (r^2 values of 0.59, 0.51, 0.57, 0.67 and 0.63 respectively). These metabolites all play integral roles in metabolic function and, as discussed above, are very good indicators of metabolic state and cell viability. Although these correlations do not provide a precise quantitative relationship, they do indicate that a great deal can be inferred about the metabolic state of harvested fish tissues from simple cut surface pH measurements. These measurements should continue to be taken in future research and represent a powerful and relatively cheap tool for use in commercial environments.

Metabolite	Pearson r	P value	r^2
Creatine	-0.70	<0.0001	0.49
Creatinine	-0.81	<0.0001	0.66
Creatine phosphate	0.77	<0.0001	0.59
Hypoxanthine	-0.41	0.0041	0.17
Uric acid	-0.57	<0.0001	0.33
Inosine	-0.38	0.0077	0.14
IMP	-0.07	0.6255	0.01
NAD^+	0.72	<0.0001	0.51
AMP	-0.03	0.8322	0.00
ADP	0.76	<0.0001	0.57
ATP	0.82	<0.0001	0.67
ATP/ADP	0.79	<0.0001	0.63
ATP/AMP	0.68	<0.0001	0.46
ATP/IMP	0.53	0.0001	0.28
K-value	-0.31	0.0295	0.10

Table 5.20 Descriptive statistics from Pearson correlation calculations showing the relationship between cut surface pH and ATP related metabolite concentrations and ratios from both rested and exhausted chinook salmon tail fillets during 30 hours storage at 15°C. Statistics in each row were calculated using 48 matched XY pairs.

5.3.6 Internal vs external fillet tissue properties after 30 hours storage

The air surrounding the fillets during storage may provide some oxygen to cells near the fillet surface and therefore allow for some aerobic ATP generation to continue. Our aim was to determine how significant this was to the metabolism of the cells near the surface. These measurements may also provide some indication as to the rate of water loss from the fillets surface.

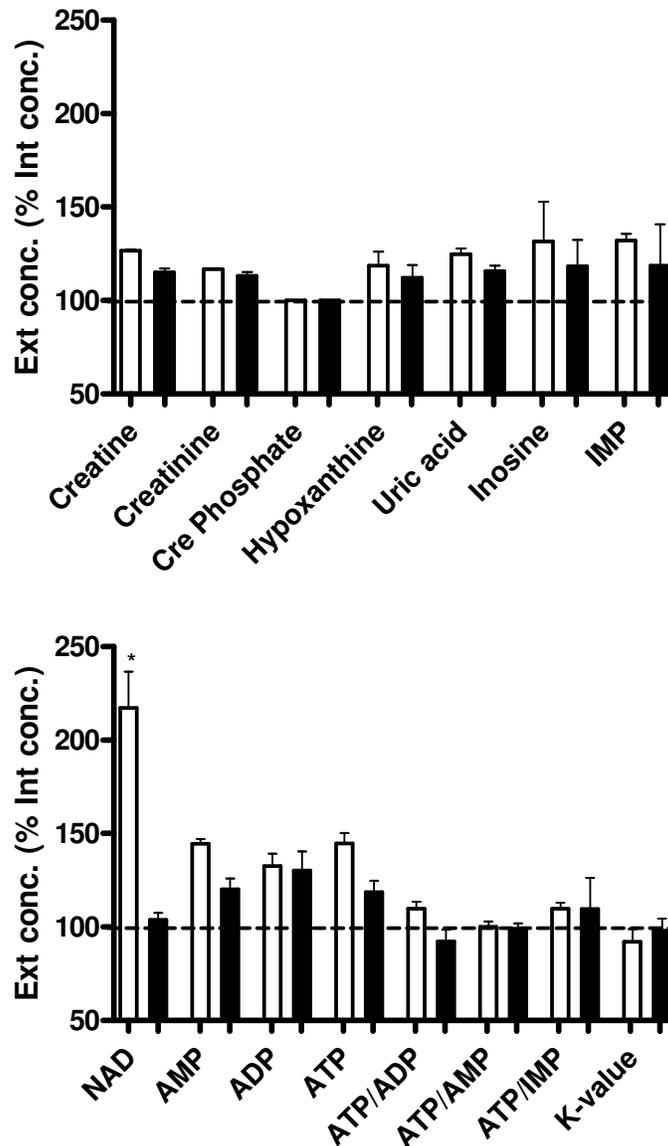


Figure 5.21 Mean metabolite concentrations from white muscle tissue samples taken from salmon fillet surfaces expressed as a percentage of matched samples from the fillet centers after 30 hours storage at 15°C in air from rested (white) and exhausted (black) fish. Results from one-way ANOVA with Bonferroni multiple comparison post-tests comparing treatments are indicated (* $p < 0.05$).

Metabolite concentrations after 30 hours of storage from both groups of fillets were consistently higher in the tissue exposed to air than in the internal tissue (Figure 5.21), although dehydration of the external tissue may have been a contributing factor, this data indicates that it does not appear to be as great as was suggested by the accumulation of isoeugenol shown in Chapter 4. The only statistically significant difference between rested and exhausted fillets was a greater difference in concentrations of NAD^+ between the internal and external tissues in rested fillets.

5.3.7 Measurement of tissue glutathione concentrations

The relationship between metabolic state and tissue GSH concentrations may indicate the role that antioxidants play during cell death and is therefore of great interest. Tissue GSH concentrations (Figure 5.22) did not change significantly during the storage period and none of the time points showed significant differences between the two harvest types. The 0 and 3 hour time points for the exhausted group were lost due to inaccurate pH buffering prior to MBB derivatization. It is very likely that technical issues associated with this technique have produced inaccurate data. This will be discussed in the following section.

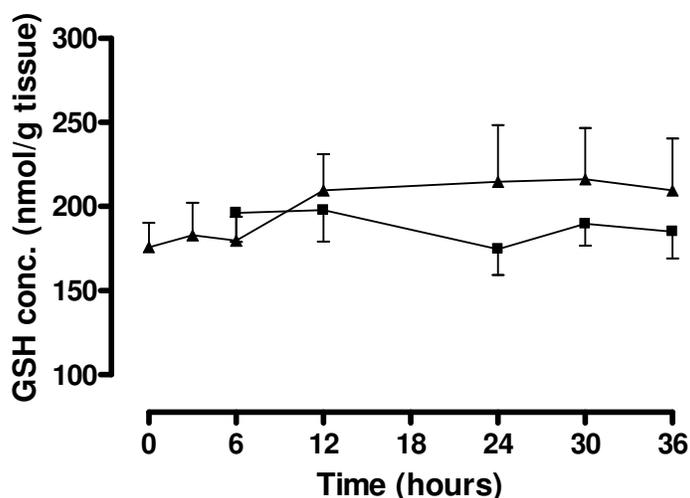


Figure 5.22 Mean glutathione concentrations ($n=4$) from the white muscle of salmon tail fillets stored at 15°C in air from rested (triangle) and exhausted (square) fish. Error bars indicate SEM.

5.4 Discussion

The cut surface pH profiles were again typical of both rested and exhausted tissues and correlated well with the concentrations and ratios of a number of ATP related compounds (Table 5.20). This clearly shows that cut surface pH provides a good estimation of metabolic state and, given the simplicity of taking these measurements, should be used routinely in future research. Notable in these data are the slightly higher cut surface pHs obtained in rested fish and the inclusion of a sampling time point after 3 hours of storage. The higher cut surface pHs reflect improvements in animal handling techniques as discussed in Chapter 2 and the extra time point allows more accurate representation of the metabolic rundown in the tissue. Generally, the trends in these data and those displayed in Chapter 4 appear comparable. The improved resolution clearly shows a period of stability from 3 to 6 hours storage, indicating that the 6 hour sampling points used in Chapter 4 produced some artificial smoothing in the data. Interestingly, this period of stability is also present in the NAD^+ and to a lesser extent the ATP and creatine phosphate profiles. Although further work with improved resolution would be required to confirm it, this is evidence that metabolic rundown takes place in a stepped manner as the cells, deprived of blood flow, adjust to new conditions to maximize survival time. This hypothesis was put forward and supported by profiles of white muscle cell membrane potentials (Janssen unpublished).

Creatine metabolism plays an important role in skeletal muscle cells. Creatine phosphate provides a source of high energy phosphate for the generation of ATP via the creatine kinase pathway ($\text{CrP} + \text{ADP} + \text{H}^+ \longleftrightarrow \text{Cr} + \text{ATP}$) (Wyss and Kaddurah-Daouk 2000; Brosnan et al. 2007). High concentrations of creatine phosphate are generated and maintained by the mitochondria in resting muscle cells from intracellular creatine and provide temporal buffering in energy supply from the sudden onset of exercise (Brosnan et al. 2007). Recently, due to its high diffusivity, views on the function of creatine phosphate as an energy buffer have shifted to one of energy translocation (Brosnan et al. 2007). Of course these functions are not mutually exclusive and the importance of each is likely to be tissue dependent. The flexibility of the creatine/creatine phosphate system in different tissues is highlighted in a review by Wyss and Kaddurah-Daouk (2000). As mitochondrial density is low (Black 2002; Tuckey 2003; Nanton et al. 2007), cytosolic creatine kinases are relatively abundant (Eppenberger et al. 1971; Grzyb and Skorkowski 2005) and cellular energy demands can change very rapidly in teleost white muscle, temporal energy

buffering in this tissue may be the more important role. The metabolic end point for creatine metabolism is creatinine, which is formed in a non-enzymatic temperature and pH dependent manner. *In vivo* it no longer takes part in the creatine/creatine phosphate cycle and is excreted in the urine (Wyss and Kaddurah-Daouk 2000).

The differences in creatine metabolism (displayed in Figures 5.4, 5.5 and 5.6) were clear between rested and exhausted chinook salmon white muscle. Rested tissue contained large quantities of creatine phosphate that was depleted after 12 hours storage. Exhausted tissue showed no detectable creatine phosphate and higher quantities of both creatine and creatinine. This shows not only a greatly reduced energy store and associated loss of energy buffering capacity in exhausted tissues, but also that the creatine cycle in general of exhausted tissues had been shifted significantly towards its metabolic end point. In rested white muscle, the rapid decrease in creatine phosphate concentrations and the rapid rise in creatinine concentrations seen after 3 hours storage shows that this system is playing a role in meeting the energy demands of post-mortem metabolism. Of particular interest is the simultaneous rundown of tissue ATP and creatine phosphate concentrations. In rested tissues, there is no indication that tissue ATP concentrations were buffered by creatine phosphate despite containing relatively high concentrations. Exhausted tissues showed complete depletion of creatine phosphate indicating the important role it played during exercise *in vivo*. Therefore the ATP buffering role typically described for creatine phosphate in exercise physiology models may not be entirely appropriate for applying to ischemic tissue models. The creatine phosphate concentrations in this work were lower than those reported in rainbow trout (McFarlane et al. 2001). This was likely caused by the transport of the fish to the laboratory and the associated delay in sampling and will have impacted both creatine phosphate and ATP concentrations.

Uric acid is the end product in the breakdown of ATP related compounds. This breakdown takes place in the following order: ATP → ADP → AMP → IMP → inosine → hypoxanthine → xanthine → uric acid (Luong et al. 1989; Carsol et al. 1997; Vazquez-Ortiz et al. 1997). Other products related to this process include hydrogen peroxide and volatile nitrogenous compounds (Luong et al. 1989; Pacquit et al. 2006). As a relatively stable metabolic end point for ATP related compounds, tissue uric acid concentrations may be indicative of freshness. Figure 5.7 shows that exhausted tissues began to accumulate uric acid immediately and rapidly. Although rested tissues also accumulated uric acid, it took place much more slowly over the first 6 hours of storage before accelerating. Rested tissue maintained significantly lower concentrations of uric acid throughout the storage

period. There were only minor differences in inosine and hypoxanthine concentrations, indicating that these relatively high storage temperatures may favour the transition of these substances through to uric acid.

It must be noted that the uric acid profiles shown here are markedly different to those seen in Chapter 4. This resulted in extensive checking of the location of the uric acid peak on the HPLC traces by spiking samples with known quantities of uric acid. For this reason I am confident that these measurements are accurate and that earlier measurements may not be. The reasons behind the loss of uric acid from the samples in Chapter 4 are not clear, but there are two potential causes. Firstly, uric acid is not particularly soluble in water and it may have been lost with the non-soluble tissue fraction that was centrifuged from the tissue homogenates prior to analysis. Also, despite being buffered from further oxidation with EDTA and BHT, the protein fractions of the tissue homogenates used in Chapter 4 were not removed or denatured. It is possible that some non-specific, unknown interactions in the homogenates were able to rapidly degrade uric acid to allantoin during the short period of time that the samples were not frozen. Given that the primary enzymes involved in uric acid metabolism are located in the liver (Cleere et al. 1976; Kinsella et al. 1985; Rumsey et al. 1991), it is unlikely that any specific enzymes were present in the tissue homogenates.

The trends in tissue IMP concentrations in rested and exhausted white muscle shown in Figure 5.10 are very different. Exhausted tissue shows high concentrations in fresh tissue that decline continually during storage. This trend has been shown in other work (Ryder 1985; Aubourg et al. 2007). Fresh rested tissue shows IMP concentrations that are much lower, but rise rapidly over the first 3 hours, and then more slowly, peaking at 12 hours. The rested tissue then maintained significantly higher concentrations of IMP for the remainder of the storage period. This may have implications for tissue quality as IMP is known to contribute to the umami-taste of fish products (Kawai et al. 2002; Kuda et al. 2007). This also indicates that rested tissues harvested from fish may benefit from a storage period creating the potential to deliver high quality fish products to customers close to the point of peak IMP concentrations.

NADH and NADPH were not detectable in these samples. It seems unlikely that concentrations of NADH would be below detection limits and it has been noted during laboratory work that both NADH and NADPH were very unstable in standard solutions. Since the collection of this data we have begun adding 5 µl/ml of 0.2 M / 0.2 M glutathione/EDTA prior to PCA extraction. This was successful in preserving previously

undetectable NADPH in cell culture samples, but unfortunately it introduced unacceptable reagent peaks. Further work to determine an appropriate reducing agent could potentially allow accurate quantification of NADH and NADPH. This would improve our ability to infer intracellular redox potential.

The cycling of NAD^+ and NADH has a wide range of biological functions. NAD^+ functions as a coenzyme for hydride-transfer enzymes and as a substrate for NAD^+ consuming enzymes (Belenky et al. 2007). NADH is a carrier of easily transferable electrons and is of fundamental importance to glycolysis (Alberts et al. 1994). As the cycling of NAD^+ and NADH is a redox reaction their relative ratios are indicators of metabolic state and intracellular redox potential (Lin and Guarente 2003). The extracellular breakdown products of NAD^+ have been shown to include ADP-ribose, inosine and uric acid (Broetto-Biazon et al. 2008). It seems likely that these processes will also occur during the post-mortem breakdown of NAD^+ in fish muscle. NAD^+ concentrations (Figure 5.1.1) during storage in rested tissue follow similar trends to those seen in creatine phosphate and ATP concentrations. Although there is a rapid reduction during the first 3 hours storage, concentrations stabilize between 3 and 6 hours. This possibly provides enough NAD^+ for dependent cellular processes to function in a reduced metabolic state. This period of stability is not present in the exhausted tissues indicating that the post-mortem metabolism is immediately and irreversibly compromised. This also indicates that the intracellular redox potential began to change very rapidly after harvesting. These changes were not shown by markers of oxidative damage such as protein carbonyls and TBARS measured in Chapter 4. This tends to suggest that oxidative damage to structural components of the cells takes place at a much later stage than modification of the intracellular redox potential. They did however correspond to the timeframes in which fluctuations in tissue vitamin C concentrations were observed in Chapter 4. Although, as vitamin C was not measured in this work, it is not possible to infer a specific relationship and tissue vitamin C concentrations still appear to be a poor indicator of metabolic state.

Recent work has shown lifespan in yeast can be extended using the NAD^+ precursor nicotinamide riboside, which functions through the elevation of NAD^+ (Belenky et al. 2007). It is very likely that maintaining intracellular NAD^+ concentrations would be a requirement for extending cell viability and/or the maintenance of a hypometabolic state in an isolated tissue.

The relationship between intracellular ATP, ADP and AMP is particularly important for maintenance of cellular homeostasis and adaptation to changing energy demands. Although ATP represents the primary source of energy, ADP, AMP and inorganic phosphate (P_i) are substrates that are not only required for generation of ATP, but are thought to play a key role in mitochondrial metabolic control (Hochachka and McClelland 1997). AMP in particular, acting through the AMP-activated protein kinase (AMPK) cascade, has been implicated in the control of a number of factors important to metabolic state and allosterically activates the glycolytic enzyme phosphofructokinase (Hardie 2000; Miller et al. 2005; Lee et al. 2006). Therefore changes in tissue AMP concentrations, although much smaller than other metabolites, may be most indicative of alterations in metabolic state. The differences in AMP concentrations shown in Figure 5.12 during the first 6 hours of storage are notable. Much higher concentrations are present in exhausted tissues and these fluctuate before stabilizing after 6 hours. Although AMP concentrations in rested tissues are lower in fresh tissue they increase over 6 hours before also stabilizing. Therefore there is the potential that elevating AMP concentrations in rested tissue immediately post harvest may slow the metabolic rundown of the tissue. This is not certain however as Horman et al. (2005) concluded that AMPK did not participate in the metabolic rate depression that occurs during ground squirrel hibernation. Changing trends were seen after 6 hours storage in rested tissue in a number of key metabolites including NAD^+ , AMP, ADP and ATP. The combination of these trends indicates that this may be very close to the end point of sustainable metabolic processes in the post-mortem tissue.

The ATP/ADP, ATP/AMP and ATP/IMP ratios (shown in Figures 5.15, 5.16 and 5.17 respectively) are likely to be more indicative of the energy state of the tissue than the concentration of any single metabolite. In rested tissue all three ratios indicate a major shift in cellular energy potential during the first 3 hours of storage. The ATP/ADP and ATP/AMP ratios indicate a slowed but continual change from 3 to 12 hours. Expressing the metabolites as ratios also removes the small period of stability present during the 3 to 6 hour storage period. This indicates that despite periods of stability in measurements such as cut surface pH and cellular membrane potentials (Janssen unpublished; Jerrett et al. unpublished), the energy potentials of the cells appear to be in a continual state of change. The stability seen in these measurements is probably due to the ion pumps responsible for the maintenance of these factors functioning at a relatively constant rate through a range of ATP concentrations.

K-values represent the ratio of hypoxanthine and inosine to the total pool of ATP related compounds and have been shown to be a reliable indicator of freshness in seafood products (Ryder 1985; Vazquez-Ortiz et al. 1997; Dondero et al. 2004; Aubourg et al. 2005a; Aubourg et al. 2007). Due to the time consuming nature and expense associated with the measurement of K-values, they have been predominantly used for research purposes and not in a commercial environment. The K-values calculated for both rested and exhausted tissues are displayed in Figure 5.18 and show that very fresh tissue has K-values very close to zero. Published K-values in fresh commercially sourced tissue tend to be in the range of 10-20 (Aubourg et al. 2007; Sallam 2007) indicating that many of the breakdown pathways have significantly progressed before experimental work began in these studies. K-values have been shown to reach 95 in coho salmon following 24 days of chilled storage and needed to exceed approximately 70 before sensory degradation was noticeable (Aubourg et al. 2007). These values are significantly higher than the maximum values seen in this work. The K-values seen in rested tissue increased at a lower rate than those measured in exhausted tissues, driven predominantly by the relative difference in IMP concentrations in the tissues. This also suggests that rested tissues have quality and shelf life benefits related to fundamental biochemical measurements over exhausted and stressed tissues. The storage temperature chosen for this work appears to favour the complete breakdown of ATP related compounds to uric acid. These processes are likely to be altered by temperature as lower storage temperatures will slow these processes and potentially promote extra pooling further up the breakdown pathway, therefore altering K-values. Further work at temperatures more representative of normal processing and storage conditions would be required to confirm the potential benefits of rested harvesting on quality and shelf life.

The mean sum of all nucleotides measured in these fillets during storage is shown in Figure 5.19. It remains within approximately 1000 nmol/g tissue of its starting point in both groups. This indicates that, although the compounds measured in this procedure are not completely contained within the breakdown chain discussed above, most remained within the chain for the duration of the storage period. This helps to validate this analytical method as a reliable means of monitoring purine biochemistry in fish tissues during storage.

The comparison between white muscle tissue from the centre and from the surface of the fillets was an attempt to identify edge effects caused by physical damage during filleting and exposure to air. As sampling from the centre of the fillets involved causing

some damage to the fillet this was done late in the storage period. The fact that all the metabolites were elevated in surface tissue indicates that dehydration may have caused most of these observed differences. However, the dehydration appears to be much less than was suggested in Chapter 4 by the accumulation of isoeugenol in the fillets. It also indicates that edge effects are not particularly large in non-viable tissue that has been stored for a long period of time. However, these effects are likely to be far more pronounced in viable tissue as the surface cells have access to atmospheric oxygen. To accurately quantify the role of edge effects as cell death occurs sampling would need to be done repeatedly during the first 12 hours of storage at 15°C.

Measurements in this work showed no difference in the reduced glutathione content of the white muscle between the two harvest groups. This is not consistent with much of the literature relating to mammalian exercise physiology (Inayama et al. 2002; Jammes et al. 2004; Steinberg et al. 2004). Although care must be taken when using these results to make inferences on fish physiology, it seems very unlikely that no changes in glutathione concentrations would take place during storage given other obvious signs of tissue oxidation. Given the nature of the data it seems likely that there are technical issues with this particular technique. Recently work by Tang et al. (2003) showed that MBB concentrations should be in excess of 200 times the total thiol content of the sample for accurate quantification. MBB concentrations in the current work were approximately 1.8 mM during derivatization and ranged between 60 and 180 times the measured GSH concentration. As this may produce a significant underestimate of tissue GSH concentrations, reducing the relative sample concentration approximately 20 fold during derivatization with MBB should maintain this ratio in excess of 200. Reliable measurements of tissue glutathione concentration in conjunction with measurements of metabolic state have the potential to further elucidate the relationship between tissue redox potential and cellular metabolic state.

5.4.1 Summary

This work has identified fundamental differences in the chemistry of chinook salmon white muscle harvested using rested and exhaustive harvesting methods. These differences continue to manifest themselves during storage of the tissue and have an effect on widely used biochemical indicators of quality. K-values increased at a significantly reduced rate in rested tissues, driven predominantly by significantly higher IMP concentrations (a factor

contributing to the umami-taste of meat products). Metabolites essential for cellular function such as NAD^+ , ADP and ATP were maintained for up to 12 hours in rested tissues. These were compromised within 3 hours in exhausted tissues. The accumulation of uric acid, the metabolic end point for many of these metabolites, was subject to a lag phase in rested tissue and remained significantly lower for the entire storage period. Manipulation of tissue AMP and NAD^+ concentrations may be useful in prolonging the metabolic rundown of rested tissues. These measurements now need to be confirmed at storage temperatures more relevant to industry and be examined for their potential to create an economic advantage in the supply of high quality seafood products.

6 - Concluding Discussion

6.1 The oxygen delivery problem

The controlled delivery of oxygen to tissues during perfusion has remained a limiting factor in advancing perfusion technologies for a significant period of time. The methods used to deliver oxygen to teleost fish muscle tissue in this work and that of Black (2002) and Janssen (2003) involve the application of high oxygen partial pressures, either through oxygen saturated saline solutions or hyperbaric oxygen treatment. These techniques showed some ability to support aerobic metabolism and, as discussed by Black (2002), hyperbaric oxygen treatment may represent a means to test the oxidative stability of tissues. This induction of oxidative stress is comparable to the chemically induced oxidative stresses that are commonly employed in cell culture studies (Giese et al. 1998; Duggan et al. 2002; Kappler et al. 2007). In relation to maintaining tissue viability, these treatments do not deliver oxygen in a manner that is analogous to the normal mechanisms of oxygen delivery *in vivo*.

The transport of oxygen both through the bloodstream and through the working cell to the mitochondria is tightly regulated. In the blood, oxygen is sequestered in red blood cells in the form of oxyhaemoglobin, and in skeletal muscle cells it is sequestered in the form of oxymyoglobin (Withers 1992). This prevents many potentially damaging oxygen based reactions from occurring and means that although the oxygen carrying capacity of the blood is high the amount of oxygen that is free in solution is very limited. Intracellular myoglobin facilitates the transfer of oxygen through the cell, serves as a short term oxygen store and buffers the release of free oxygen to the mitochondria (Withers 1992). This means the total concentration of oxygen free in solution in the intracellular fluid is also low and maintained almost constant up to the maximum sustainable aerobic metabolic rate despite large changes in flux (Hochachka and McClelland 1997). As the relationship between work and oxygen delivery is almost always 1:1 for aerobic metabolism, Hochachka and McClelland (1997) have postulated that oxygen delivery plays a key role in metabolic control.

In this light the finding that oxygen saturated perfusion saline was causing oxidative damage to aerobic skeletal muscle tissue in the perfused salmon tails (Figures 3.2 and 3.4) was perhaps not surprising. As damage was evident in the tissues it also raises the

possibility that oxidative damage was also occurring in the vasculature. Any damage to the vasculature would ultimately limit perfusion performance and this may have played a role in the poor perfusion of the white muscle tissue in the salmon tails. Many of the laboratory grade chemicals used in the perfusion saline contain trace quantities of transition metals and it has been shown that these can contribute to tissue damage in perfused isolated rat hearts (Powell and Wapnir 1994). Therefore there is the potential for Fenton chemistry to contribute to and/or initiate oxidative damage in both the tissue and the vasculature of the salmon tails. Further investigation of the redox potential of the perfusion saline and comparison to teleost blood plasma may be warranted. Examining the formation of protein carbonyls in the oxygen saturated saline during pre-perfusion storage would help to address whether Fenton chemistry can occur in the isolated saline or whether it is only an issue once inside the salmon tail preparation. Although attempts were made to measure oxidation in the perfusion saline in the early stages of this research, samples were only taken as it exited the preparation, and TBARS and protein hydroperoxide concentrations were used to indicate oxidation. These showed no signs of increasing over the perfusion period. In hindsight measuring protein carbonyl concentrations, generated through the oxidation of the BSA in the oxygen saturated saline, over the course of the perfusion period before it was pumped through the preparation may have been more appropriate. Regardless of the location of action, the addition of transition metal chelating compounds to perfusion salines has shown the ability to inhibit iron mediated oxidation (Rauen et al. 2007) and should significantly reduce the oxidative damage to future perfusion preparations.

A controllable method of oxygen delivery that creates conditions in the saline comparable to those found in the blood will need to be used before major advances in preserving isolated tissues with perfusion will be feasible. Maintaining endogenous antioxidant function and intracellular redox potential will almost certainly be necessary to allow the long term maintenance of metabolic function in isolated tissues, but at this time an appropriate form of oxygen delivery is the issue of primary importance. Addressing this issue will provide two key benefits. Firstly, it will allow a further mechanism of control over metabolic rate, and secondly it will limit any vascular and tissue damage caused by high oxygen partial pressures. This will both reduce the need to control damaging oxidative reactions and allow the addition of antioxidant compounds that are susceptible to oxidation to the perfusion saline to further stabilize the tissue and support the continued function of the endogenous antioxidant systems. The use of red blood cells in

the perfusion saline has been shown to improve oxygen delivery in our laboratory (Forgan unpublished), but it is somewhat inconvenient keeping the red blood cells in suspension and the shelf life of the saline solution is limited. Therefore some obvious candidates include the haemoglobin based oxygen carriers currently under development. The length of time taken to develop these compounds reflects the serious technical challenges involved. However, a recent review by Diesen and Stamler (2007) discusses the promising results of *S*-nitrosylated polyethylene glycol modified haemoglobin (SNO-PEG-Hb). This compound has showed effective function as an oxygen delivery compound *in vivo* while mitigating the vasoconstrictive action of haemoglobin and allowing better regulation of vascular tone. Although these compounds are being developed primarily for medical use as blood transfusion substitutes they have the potential to greatly contribute to tissue preservation techniques and therefore future developments should be closely monitored.

6.2 The limits of the antioxidant solution

Antioxidants, both synthetic and naturally derived, have repeatedly been shown to offer shelf life and product quality benefits in a number of meat and seafood products (Halliwell et al. 1995; Tozer 2001; Banon et al. 2007; Goulas and Kontominas 2007). In this particular application these compounds serve to prevent oxidative processes, particularly in the lipid fraction, that can produce rancid odours and flavours in the meat. This role, although important for improving food processing and storage technology, is not necessarily relevant to maintaining the viability of the cells within the tissue. Antioxidants have also been included in saline solutions for organ preservation. Examples include the University of Wisconsin buffer and the more recently developed Polysol (Bessems et al. 2005a; Bessems et al. 2005b). These applications involve relatively large concentrations of glutathione (which can be easily oxidized in solution and therefore the quantity of reduced glutathione that ultimately reaches the tissue may be low) and allopurinol. Although promising results have been gained in maintaining rat and pig livers with Polysol (Bessems et al. 2005a; Bessems et al. 2005b; Bessems et al. 2006), the contribution of the antioxidants in the saline to this success is uncertain. However, perfusion of rat livers with the mitochondrially targeted antioxidant MitoQ reduced ischemia-reperfusion injury, clearly indicating the importance of antioxidant function in maintaining both mitochondrial function and cell viability during the storage of tissues (Davies et al. 2002).

These successful applications are contrasted by work, including that discussed in Chapter 3, that show certain antioxidant applications do little to reduce oxidative damage (Morrissey et al. 1998; Hamre et al. 2003; Ruff et al. 2003). As antioxidant compounds have specific modes of action, these findings may be due to inadequate matching between the mode of oxidative damage and the antioxidant used. Also, as a complex balance exists *in vivo* between antioxidant and pro oxidant compounds, the application of only one or two antioxidant compounds may be inadequate to support endogenous antioxidant function, particularly in the case of viable tissues. This introduces a need for relatively complex antioxidant solutions. The tissue preserving solution Polysol contains high concentrations of glutathione (3 mmol/L) and allopurinol (1.2 mmol/L), and lower concentrations of α -tocopherol and ascorbic acid (combined concentration of 0.15 mmol/L) (Bessemers et al. 2005b; Bessemers et al. 2006). This is likely to provide relatively broad protection from a range of oxidative processes. However, the need to include these particular concentrations appears to show the evolutionary development of Polysol from the earlier developed University of Wisconsin buffer. More specific data regarding the stability and/or interaction of these antioxidants with the preserved tissue would be of interest.

A potentially promising solution to the need for complex antioxidant solutions is to use plant extracts as these contain many antioxidants, potentially at functional relative concentrations. This approach has shown potential in inhibiting oxidation in food products (McCarthy et al. 2001; Goulas and Kontominas 2007). Some plant extracts have also been shown to have vasoactive properties (Dell'Agli et al. 2004; Runnie et al. 2004; Ghayur et al. 2005). Very little work has been published on the contribution of plant extracts to isolated tissue perfusion models, but certain plant extracts have shown hypotensive effects in anaesthetized rats and perfused rat hearts (Tahri et al. 2000; Consolini and Sarubbio 2002; Consolini and Migliori 2005). Examining the effects of plant extracts in perfusion models, particularly their potential to enhance perfusion performance and contribute to the oxidative stability of the tissue and/or endogenous antioxidant function is a possible area of future research. Although this holds potential for food processing technologies, any findings of interest may not be easily transferred to organ preservation applications as there is a need in this case for all the compounds entering the tissue to be known and strictly controlled.

Working with whole tissues in perfusion models introduces some uncertainty as to the relative locations of the antioxidants delivered to the tissue through the perfusion saline. In particular there is the question of whether they will cross cell membranes or

remain in the blood vessels and interstitial spaces. As the analytical techniques used in this work to measure antioxidant compounds such as ascorbic acid measure extracted tissue averages, they are not useful for determining relative locations *in vivo*. In the case of perfusion models the ability of ascorbic acid to enter the cells is dependent on the assumption that cell membrane transporters remain functional in the perfusion environment. Also, as not all antioxidant compounds have specific transporters on the cell membrane, the effectiveness of water soluble antioxidants in a perfusion environment may be limited by an inability to enter the cells. This may partially explain the lack of protection afforded to the red muscle of the salmon tail perfusion discussed in Chapter 3 (Figures 3.2 and 3.4) despite high concentrations of uric acid in the perfusion saline. This lends favour to the concept of using small lipid soluble antioxidant compounds such as isoeugenol and/or other phenolic compounds as it is more likely that they will be able to cross cell membranes.

There is a great deal of future promise in the field of using antioxidant compounds to preserve food products and the viability of isolated tissues. However, there is also a limit as to how effective they can be. For example, although endogenous antioxidant systems have been shown to be up regulated in response to oxidatively stressful events such as exercise (Gomez-Cabrera et al. 2007; Ji 2007; Jackson 2008; Ji 2008; Sachdev and Davies 2008), exercise of extreme duration or intensity can cause tissue damage (Mastaloudis et al. 2001; La Gerche and Prior 2007). This indicates that the endogenous antioxidant system cannot be infinitely scaled and that even with appropriate support there is a limit to the amount of oxidative stress that can be endured in any biological system. Therefore attempts to control oxidative stress in perfusion models must focus on both limiting the causes of oxidative stress and supporting antioxidant function. In terms of the salmon tail perfusion model used in this work this again raises the issue of using appropriate forms of oxygen delivery to minimize oxidative stress.

6.3 Model tissues for the study of hypometabolism

A number of animals have shown the ability to tolerate hypoxic conditions, and therefore the physiology and biochemistry of the relevant biological mechanisms, both in the whole animal and individual tissues is of great interest scientifically. However, these mechanisms are complex, tissue specific and highly regulated (Hochachka et al. 1996; Hochachka et al. 1997; Boutilier 2001; Hochachka and Lutz 2001; Storey 2002; Milton

and Prentice 2007). Teleost white muscle tissue is somewhat hypoxia tolerant due to its high anaerobic potential (Kiessling et al. 2006), and not due to an ability to reduce energy consumption and carefully match energy demand to the limited supply during hypoxia. This allows cell viability to be maintained for a period of time, but without aerobic recovery it is not able to sustain the tissue long term. A reliance on anaerobic energy production during hypoxia also alters the acid-base balance of the cells and ultimately serves to limit tolerance times (Jackson 2004). Strategies used by hypoxia tolerant animals to control acid-base disturbances include sequestering lactate, releasing bone minerals to provide increased extracellular buffering capacity and using alternative metabolic pathways to produce ethanol instead of lactate (Jackson 2004). These strategies are not available to hypoxia sensitive animals and/or tissues.

The cellular processes responsible for the majority of the energy demand in hypoxia tolerant turtle hepatocytes include protein synthesis, protein degradation, Na^+/K^+ pumping, urea biosynthesis and glucose biosynthesis (Hochachka et al. 1997; Hochachka and Lutz 2001). Apart from urea and glucose biosynthesis these energy consuming processes are comparable to skeletal muscle. During anoxia in turtle hepatocytes, protein turnover is reduced to approximately 10% of the normoxic rate, urea and glucose biosynthesis cease and Na^+/K^+ ATPase activity is reduced to approximately 25% of the normoxic rate and becomes the major energy consuming process in the cell (Hochachka and Lutz 2001). This is accompanied by a similar reduction in membrane permeability, which, even during normoxia is relatively impermeable compared to rat hepatocytes (Hochachka and Lutz 2001). This allows normal electrochemical gradients to be maintained. This raises important concerns about whether inducing a hypometabolic state in a tissue that does not normally experience such events *in vivo* is feasible. The fact that normal cellular conditions are maintained during periods of hypoxia in hypoxia tolerant animals means that care needs to be taken when assessing the relative success of attempts to prolong cell viability. Periods of stability in some parameters of cellular condition below what is generally regarded as normal may provide an opportunity to stabilize the cells in that state, or it may simply be a transient and unsustainable phase that inevitably leads to cell death. This is an issue that requires further investigation.

The specific properties of turtle hepatocytes, in particular the membrane permeabilities and subsequent changes in membrane permeability in response to hypoxia, make applying knowledge gained from this example to tissues such as teleost skeletal muscle difficult. This is further complicated by the performance uncertainties (e.g. oxygen

delivery rates) inherent in the perfusion model used in this work. Also, as salmon are generally not particularly hypoxia tolerant (Waller et al. 1997), it may be inherently difficult to induce a hypometabolic state in salmon tissues, particularly through perfusion. The goal of inducing and maintaining a hypometabolic state in tissues that do not normally experience such events is certainly a worthwhile endeavor, but achieving measurable success may firstly require some less ambitious goals. Future work attempting to induce hypometabolism through perfusion in a hypoxia tolerant teleost (or other animal) may provide a particularly useful model. This would allow the comparison of measurements of metabolic state in tissues from whole animals during hypoxia to those taken during isolated perfusion. This would provide a quantitative measure of success and also eliminate a number of concerns over the feasibility of such work. The accurate characterization of metabolic state during hypometabolism would also provide an accurate goal for future work in non-hypoxia tolerant tissues.

Signaling pathways for inducing hypometabolism or slowing metabolic rate are also of interest. The extracellular concentrations of compounds such as adenosine have been shown to increase significantly during hypoxia in turtles and it may play a role in reducing protein turnover and membrane permeability (Buck 2004). Therefore adenosine concentrations in the range of 20 μM in the perfusion saline, similar to those found in the intracerebral spaces in the turtle, may potentially help to improve the performance of perfusion preparations. Hydrogen sulfide (H_2S) has shown a promising ability to reduce oxygen consumption and body temperature in mice (Blackstone et al. 2005) and may therefore be a useful tool for controlling metabolic rate. H_2S has also been shown to inhibit hypochlorous-acid mediated oxidative damage in human cell lines (Whiteman et al. 2005). However, the significance of this in relation to maintaining cell viability and preserving fish tissues is uncertain. Recent work has also shown that the reduction of metabolic rate caused in mice by H_2S exposure does not occur in sedated sheep (Haouzi et al. 2008). This highlights the need for caution when applying scientific findings across species barriers.

The potential applications of studying tissues that are known to undergo hypometabolic episodes *in vivo* have long been recognized (Hochachka and Lutz 2001; Buck 2004). Although applying principles used by hypoxia tolerant tissues to the preservation of tissues that are normally hypoxia sensitive may prove challenging, this approach may significantly contribute to practical knowledge and techniques in the field of tissue preservation.

6.4 Measuring metabolic state

This work, using both the perfused salmon tails and the isolated tail fillets, was made difficult because of an inability to accurately relate the protein and lipid oxidation data to a reliable measure of metabolic state in the respective tissues. It was this issue and the notable accumulation of uric acid (the metabolic end point for a number of ATP related compounds) in stored tail fillets that led to the adaptation and implementation of the HPLC assay based on that published by Furst and Hallstrom (1992). This allowed measurement of all of the purine bases, nucleotides, nucleosides and creatine bases in the tissues. The data collected from salmon tail fillets stored at 15°C clearly showed a rapid metabolic rundown in both rested and exhausted tissues. This was indicated by the rapid decrease in the concentrations of some key metabolic compounds including creatine phosphate, ATP, ADP and NAD⁺ (Figures 5.6, 5.14, 5.13 and 5.11 respectively). As the generation of ATP via both aerobic and anaerobic pathways requires adequate intracellular concentrations of ADP and NAD⁺, the depletion of one of these compounds could result in the loss of metabolic function despite the presence of adequate concentrations of oxygen. The ability to measure all of these compounds provided us with a high degree of certainty in making inferences about metabolic state.

Previous work by Black (2002) showed that aspects of teleost muscle metabolism were supported by hyperbaric oxygen treatment, but that aspects of mitochondrial function may have also been damaged. Black (2002) suggested that a combination of factors was likely to have led to the loss of cell viability including the build up of metabolic waste products and damage to the mitochondria by high oxygen partial pressures. Given that the tissue was ischemic there is the strong possibility that the depletion of important metabolic substrates may also have been a contributing factor. The lack of information in Black's work regarding the relative concentrations of metabolic substrates such as ADP, AMP and NAD⁺ in the respective tissues means that a clear picture of the relative metabolic states was not obtained. This work, although completed under different experimental conditions, clearly shows that tissue NAD⁺ and ADP concentrations rapidly decline after 6 hours of storage at 15°C (Figures 5.11 and 5.13). This rapid decline may be indicative of an important transition in the onset of cell death in the tissue. Maintaining ADP and NAD⁺ concentrations will be a likely requirement for maintaining cell viability and the relative success of attempts to do so therefore require evidence that this has been achieved.

The work presented here clearly shows that waste products from purine metabolism, in particular uric acid and hypoxanthine, began accumulating in the tissue immediately following ischemia (Figures 5.7 and 5.8). This occurred before depletion of metabolites such as ATP and is typical of stored fish products (Luong and Male 1992; Carsol et al. 1997; Aubourg et al. 2007), but does not normally occur *in vivo*, even following exhaustion. Although the ability to remove these waste compounds through perfusion has not been directly examined in this work, reducing or eliminating the accumulation of uric acid and hypoxanthine is likely to be important for future improvements in extending cell viability in isolated tissues.

Although the measurement of absolute tissue concentrations of metabolites does not directly provide information on metabolite turnover rates, Hochachka and McClelland (1997) have pointed out that when a tissue is in homeostasis, the changes in concentration of metabolically important compounds such as ATP and oxygen are very low. Therefore significant changes in metabolite concentrations do provide some information regarding metabolite turnover. Inducing a state of stable metabolite concentrations in a perfused tissue would clearly indicate some success in attempts to maintain cell viability. Also, rapid changes in metabolite concentrations could be used as an early sign of the onset of processes leading to cell death. Robust measures of metabolic state and metabolite turnover rates have the potential to contribute greatly to future research in tissue preservation.

Although the HPLC assay used in this work to measure ATP related compounds is complex and the run time is long, the automated nature of modern HPLC equipment means that it is feasible for use in a number of research settings. The information generated is directly relevant to the study of cell metabolism and death and can also be used to infer product quality when applied to the processing and storage of meat and seafood. It has also been shown to be useful in estimating the age of fish products (Vazquez-Ortiz et al. 1997). Even in circumstances where metabolic chemistry is not directly of interest, knowledge of metabolic state could provide useful background information when interpreting data. The use of this technique, or others that provide similar data, is important particularly in the field of tissue preservation.

6.5 The role of AQUI-S™ in preserving tissue

Isoeugenol, the active ingredient of AQUI-S™, has a number of properties that are of interest and may contribute to its ability to preserve tissue. The greatest contribution is undoubtedly its anaesthetic properties, which allows the harvesting of seafood in a rested physiological state (Jerrett and Holland 1998; Iversen et al. 2003; Roth et al. 2006; Bosworth et al. 2007). This action is mediated via the formation of a competitive blockade of neuromuscular transmission (Ingvast-Larsson et al. 2003). Also, eugenol has been shown to alter membrane permeability in gram-negative bacteria (Gill and Holley 2006) and this finding may have some significance regarding the effects of isoeugenol on muscle cells. Although the concentrations of eugenol used by Gill and Holley (2006) are many times higher than those achieved by isoeugenol in rested teleost white muscle, the membrane properties of gram-negative bacteria are very different to those of muscle cells and may require much higher concentrations for this effect to be measurable. Also, due to its lipophilic nature, the localized concentrations of isoeugenol in and around the cell membranes of the muscle cells may be many times greater than the measured average tissue concentrations suggest. This possible action requires further investigation as alterations in membrane permeability are heavily involved in tissues known to undergo prolonged periods of hypometabolism (Hochachka and Lutz 2001; Milton and Prentice 2007).

The physiological effects of AQUI-S™ include slowed ventilation rates and reductions in heart rate, cardiac output, dorsal aortic pressure and stroke volume (Hill and Forster 2004). Given these conditions it is likely that some tissues undergo hypoxia during anaesthesia, and therefore repeated anaesthesia may induce a conditioning response. Ischemic pre-conditioning has been shown to elicit a preserving effect in a number of tissues including pig skeletal muscle (Pang et al. 1995; Badhwar et al. 2004; Pasupathy and Homer-Vanniasinkam 2005). Catalase was found to prevent this conditioning response in cardiac muscle indicating the importance of reactive oxygen species as signaling agents (Facundo et al. 2006). The potential for repeated anaesthesia with AQUI-S™ to elicit a conditioning response may be important for two reasons. Firstly, this could be an easy technique to condition animals for harvesting, potentially maximizing the protective effects induced by using AQUI-S™. However, this also means that caution may be needed when making inferences about the effects of AQUI-S™ as the tissues of laboratory animals that are repeatedly anaesthetized may not be directly comparable to those that undergo

anaesthesia infrequently as is likely in an aquaculture environment. This possible effect will have been limited in this work as the animals were generally anaesthetized only once.

The benefits of rested harvesting were clearly shown in Chapter 5 by the slowed depletion of metabolically important compounds and the reduced accumulation of waste products from purine metabolism. Although the storage conditions were not typical of those used in industry, there was clear evidence that quality and shelf life were improved by rested harvesting procedures. In contrast, exhausted tissues showed signs that cell viability was compromised during harvesting by excessive stress and exhaustion and this resulted in reduced fillet quality and shelf life. This certainly suggests further research using industry relevant storage conditions would be appropriate. In regards to tissue preservation research, it also clearly indicates that the chances of successfully maintaining cell viability are greatly increased by working with tissues that are removed from the animal in a rested state.

The use of AQUI-STM during the harvesting procedures, regardless of the metabolic state of the tissue, significantly inhibited lipid peroxidation in the white muscle of tail fillets stored in air at 15°C (Figure 4.2). This, and the fact that lipid oxidation occurred at a stage when the tissue was unlikely to be viable, strongly suggests that the chemical properties of isoeugenol are responsible for this effect and not the different respective metabolic states of the tissues. Isoeugenol is a lipophilic compound that is known to have antioxidant properties both through its action as a chain-breaking antioxidant and its ability to chelate some transition metals (Brandwilliams et al. 1995; Davcheva et al. 1995; Uchida et al. 1996; Cuvelier et al. 2003; Ito et al. 2005). It has also shown antimicrobial properties (Mansour et al. 1996), but rested harvesting did not reduce microbial counts in channel catfish fillets (Bosworth et al. 2007) and therefore this is unlikely to be a relevant property in terms of its tissue preserving potential. As isoeugenol is lipophilic it is likely that it is strongly associated with the lipid fraction of the tissue and the interaction between the aqueous and lipid fractions of non-viable tissues is probably limited. This is supported by the observation that isoeugenol did not reduce protein oxidation rates in stored salmon tail fillets (Figure 4.3). The Fenton and Haber-Weiss reactions are likely to play significant roles in initiating oxidative processes in non-viable tissues due to the release of redox available iron from intracellular myoglobin stores (Morrissey et al. 1998; Baron and Andersen 2002; Chaijan et al. 2005). Also, isoeugenol concentrations did not decrease in the stored tissues (Figure 4.8) as would be expected if it was functioning predominantly as a chain-breaking antioxidant. This suggests that the antioxidant action of isoeugenol in the

lipid fraction of teleost white muscle tissue may be predominantly mediated via its transition metal chelating property.

In summary, the contribution of AQUI-S™ to preserving tissue viability is primarily via its action as an anaesthetic allowing harvesting of rested tissue. There is a possibility that associated changes in membrane permeability may be relevant to this action, but it does not appear that its antioxidant properties are. Repeated anaesthesia may also induce a hypoxic conditioning response. The antioxidant action of AQUI-S™ is specific to the lipid fraction of the tissue and may be primarily mediated through its transition metal chelating activity. This serves to effectively inhibit late stage lipid oxidation in non-viable teleost white muscle. Further work is required to investigate any further commercial relevance of these findings.

6.6 The use of preserving compounds during the harvesting and processing of seafood

The data from Chapters 4 and 5 of this thesis clearly show that the use of AQUI-S™ during the harvesting of salmon has preserving effects on the tissue, and that this is likely to be relevant to a range of storage conditions above freezing. The ability to introduce preserving compounds into seafood products through the water prior to and during harvesting represents an important proof of principle. The ease with which compounds can be delivered to tissues in this way is likely to be related to the size and relative hydrophobicity of the compound due to the requirement that it crosses the gill epithelia. However, it has been shown that the ascorbic acid concentration of channel catfish fillets could be elevated through exposure of the whole animal to 0.3% sodium ascorbate in the water pre-harvest (Thed and Erickson 1992). This indicates that a wide range of compounds could potentially be delivered to muscle tissues in this manner. This approach to introducing preserving compounds into harvested tissues has significant advantages, particularly where the tissue is maintained in its original form (i.e. fish fillets or whole shellfish as opposed to mince), as it uses the animals natural circulatory system to deliver compounds throughout the tissue. Although endogenous antioxidant concentrations in tissues can be modified via the diet (Gatlin et al. 1992; Sigurgisladottir et al. 1994; Jensen et al. 1998), exogenous antioxidants administered in this manner are not effective (Gatlin et al. 1992) and may be rapidly broken down *in vivo*. Most other attempts to manipulate the oxidative stability of the tissue use post-harvest techniques such as soaking or rinsing

with antioxidant solutions and/or modified atmosphere packaging (Richards et al. 1998; Fagan et al. 2004; Goulas and Kontominas 2007; Sivertsvik 2007). Although some success has been reported here the effects are likely to be predominantly on the tissue surface.

Polyethylene glycol and the amino acid glycine have shown promise in preserving tissues (Churchill et al. 1995; Arora et al. 1999; Ascher et al. 2001; Badet et al. 2005; Ben Mosbah et al. 2005; Baptiste and Fehlings 2006) and may be able to be delivered to tissues through the water during harvest. These compounds, or others with similar properties, could potentially help to stabilize the tissue and reduce cold damage during storage. Plant extracts have also shown an ability to inhibit oxidation in meat products (Mielnik et al. 2003; Banon et al. 2007; Raghavan and Richards 2007) and those containing high concentrations of resveratrol or related compounds may offer other preservation benefits (Plin et al. 2005). Using plant extracts in preserving techniques is also attractive as it likely to be an acceptable practice to consumers, unlike many synthetic compounds (Lindberg Madsen and Bertelsen 1995).

The delivery of preserving compounds to aquacultured seafood through the water prior to or during harvesting is a promising avenue for future research. Ultimately there is the potential for products like AQUI-S™ to be tailored specifically to particular aquacultured species and/or the specific storage requirements of the end product.

6.7 Concluding remarks

The field of tissue preservation has been, and will remain for the foreseeable future, a challenging one. Despite this a great deal of progress has been made, motivated by the many potential industrial and medical applications and the associated economic benefits. In particular, this work has shown that the development of reliable oxygen carrying compounds that mimic the function of red blood cells is an unavoidable requirement before significant progress can be made. This will mark a significant milestone and hopefully allow further rapid progress to be made.

The important role of antioxidant compounds in supporting metabolic function is undeniable. However, although their potential to inhibit oxidation in meat and seafood products during storage has clearly been shown, an antioxidant containing solution specifically to support metabolic function in viable tissues has not yet been designed. The inability of antioxidants to provide protection from oxidation in certain circumstances also

highlights the need to tailor antioxidant solutions to the specific requirements of the application. Improved analytical techniques for measuring specific and general oxidation products and antioxidants, and also the development of tools to measure the redox potentials of both the intra and extracellular fluids in viable tissues would greatly benefit future research efforts.

AQUI-STM has been shown to have some significant effects on the white muscle of chinook salmon, both immediately post-harvest and during storage. Some of these are related to its antioxidant function, and more specifically may be mediated by its ability to chelate transition metals. These effects are likely to be relevant to most other seafood and meat products, but the commercial significance of these findings are unknown. AQUI-STM has proved to be a valuable research tool and has the potential to significantly contribute to the aquaculture industry.

There is a great deal of potential for future research aiming to preserve tissues for human consumption. In particular it would be valuable to determine the relationship between iron release and tissue oxidation and the potential to use iron chelators including plant derived flavonoids as a means of preserving tissue. Other processes involved in tissue degradation such as lysosome activation could also potentially be influenced to help maintain tissue integrity and structure. It is certain that research in the fields of physiology and biochemistry will play a significant role in advancing tissue preservation technologies.

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