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Protein and lipid oxidation in fish: pathways, kinetics and products
Nima Hematyar
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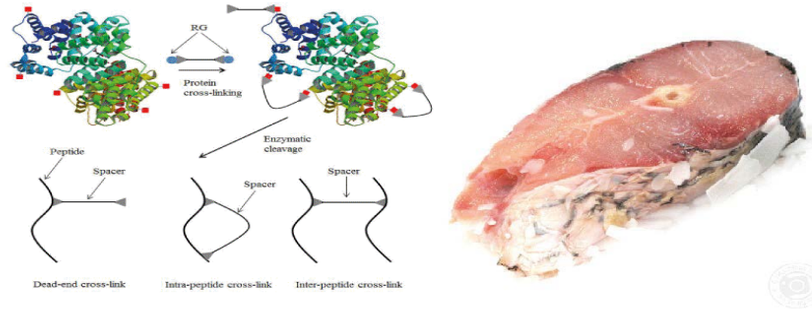

Nima Hematyar

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

Effects of lipid and protein oxidation on fish muscle quality

According to the FAO (2016) report, the annual fish consumption till 2014 in the world was around 20 kg per capita. On the other hand, lower fish consumption have been reported for Central European countries. FAO reported, an increasing trend of aquaculture production during last 25 years which can provide 50% produced fish for consumption (FAO, 2014).

Fish muscle has an excellent nutritional value which can provide a high proportion of unsaturated fatty acids and good quality protein along with vitamins and minerals. Generally, fish lipids have important nutritional values, particularly owing to the high proportion of n-3 polyunsaturated fatty acids (PUFA) (Kjaersgard, Norrelykke, & Jessen, 2006). Several studies have confirmed a relationship between the consumption of PUFA and reducing the risk of some diseases, such as stroke and heart diseases (Zhang, Sasaki, Amano, & Kesteloot, 1999). For instance, the importance of docosahexaenoic acid (DHA, C22:6n-3) for infant's brain development as well as its role for the maintenance of adults brain have been reported before (Horrocks and Yeo, 1999). Additionally, due to the high digestibility, fish protein as a food is greatly recommended. Fish, in general, is a highly perishable commodity and the same applies for value added commodities derived from fish.

On the other hand, consequences of post-mortem alteration in fish muscle during storage lead to change the fish fillet quality and nutritional value therefore, it is important to investigate post-mortem phenomena. Oxidation and the microbial spoilage are important factors in food deterioration that can affect the shelf life and the quality of food products (Ashie, Smith, & Simpson, 1996). Oxidation is initiated by various factors such as light, heat, reactive oxygen species (ROS), and oxidation promoters. In the human metabolism, ROS seems to be associated with many diseases, like autoimmune disease and inflammatory disorders (Bodamyali, Stevens, Blake, & Winyard, 2000). ROS causes important damage such as denaturation of proteins, peroxidation in lipids, membrane destabilization, and DNA mutation, which may result in some serious diseases like cancer (Borchani, Besbes, Masmoudi, Blecker, Paquot, & Attia, 2011). Additionally, oxidation leads to several changes in food products quality, which can decline the acceptability of them for costumers. The process of oxidation during the storage of foods that is caused by ROS concerns both lipids and proteins (Bernardini et al., 2011). For many years, lipid oxidation development has been investigated intensively because of its effects on odour and taste. In addition, the measurement of lipid oxidation products (hydroperoxides, hexanal, and malondialdehyde) is much easier than analysing the protein oxidation products. Compared to lipids, proteins were ignored for several decades as a target of oxidation. Due to the 3D-structures of proteins, when oxidation happens, it leads to several alterations in both chemical and textural parameters. In general, the same factors causing lipid oxidation will also cause protein oxidation. Furthermore, the kinetics and pathways of protein oxidation are similar yet more complex than lipid oxidation.

As touched upon above, high amounts of omega 3 fatty acids in fish fillets can prevent several diseases; however, regardless of nutritional value, the high amount of PUFA and the presence of some pro-oxidants, such as heme groups, make fish muscle prone to oxidation (Richards & Hultin, 2002). Processes like autoxidation, enzymatic catalysed oxidation and photosensitized oxidation are three major mechanisms in lipid oxidation. Autoxidation in meat and fish can be initiated by light, heat, and the presence of metal ions and radicals. Very low concentrations of radicals are needed to start the reaction. In fish muscle especially, after death metal catalysis can be marked as an important lipid oxidation process which is started by converting ferrous (+2) to ferric (+3). When the autoxidation process is started, a cascade of reactions will happen and each new molecule can enhance the variability and reaction speed (Heinonen, Meyer, & Frankel, 1998). Sensory parameters, nutritional value,

and colour are the main quality characteristics that can be influenced in muscle-based foods by the progress of lipid oxidation. Lipid oxidation in fish leads to off flavour, rancid taste, and development of several different substances which have toxic effects on human health (Medina, González, Iglesias, & Hedges, 2009). Additionally, sensory quality parameters in relation to oxidation development were assessed in our study on carp fillet in chapter 3. The result indicated a decreasing trend of common carp fillet acceptability by the effect of oxidation progress. Moreover, lipid oxidation limits the shelf life and thereby influences on the fish and fish products marketing. For these reasons, it is important to know well about the mechanisms and products of lipid peroxidation beside the contribution of lipid oxidation on the final quality of fish muscle. Therefore, monitoring lipid oxidation development in fish fillets is important to find a way for retarding the oxidation progress.

Coming to protein oxidation, it is caused directly by ROS and also reactive nitrogen species (RNS) or indirectly through the reactions between products of lipid oxidation with carbohydrate or reducing sugars (Lund, Heinonen, Baron, & Estevez, 2011). Sites of oxidant damage on proteins include the backbone (protein cross-linking and cleavage peptide bonds) and protein side chains. However, amino acids side chains are the main oxidative modifications of proteins which lead to formation of carbonyl groups, thiol oxidation and aromatic hydroxylation (Stadtman, 1990). The most prominent result of metal ion-catalysed oxidation of myofibrilar protein is the formation of carbonyl compounds with amino acid side chains (Levine, 1984). In some proteins, metal ion-catalysed oxidation systems can easily oxidize the side chains of amino acid residues. Lysine, proline and arginine are the most important targets of metal catalysed oxidation, and glutamic semialdehyde is also an important product of this reaction (Amici, Levine, Tsai, & Stadtman, 1989). Additionally, consequences of backbone protein oxidation leads to protein fragmentation and alteration of secondary and tertiary of the protein structure. This kind of oxidation causes protein aggregation and polymerization by formation of disulfide, and dityrosine bridges (Martinaud et al., 1997). Due to the presence of reactive sulfur atoms cysteine and methionine are the most prone amino acids to oxidation (Shacter, 2000). In general, formation of protein hydroperoxides and protein carbonyl groups are the most common amino acid modifications, whereas cross-linking has been explained as the formation of disulphide and di-tyrosine through the loss of cysteine and tyrosine residues (Estévez, Ollilainen, & Heinonen, 2009). The significance of protein oxidation and subsequent impact on fish muscle quality during the storage period has been investigated earlier (Bertram et al., 2007; Lund, Hviid, & Skibsted, 2007; Ooizumi & Xiong, 2004). Several studies have proved that consequences of protein oxidation lead to protein structure changes and also chemical alterations on individual amino acids and losing their functionality (Badii & Howell, 2002; Dalsgaard, Nielsen, Brown, Stadler, & Davies, 2011; Herrero, Carmona, & Careche, 2004). Due to protein oxidation, the water holding capacity (WHC), firmness of muscle, and solubility of proteins are reduced. Also, the amount of sulfhydryl groups is decreased. Myosin and actin proteins are vulnerable to oxidation, particularly myosin heavy chain (MHC) owing to the presence of cysteine in the root tail, which is responsible for protein cross-link (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010). Jasra, Jasra, & Talesara (2001), reported the denaturation of myosin light chain (MLC) and α-actin with 1D gel electrophoresis in the carp (*Labeorohita*) muscle after 6 months storage at -20°C. According to the western blot analysis, Baron et al. (2007) reported that proteins in two different weights 200 and 38 kDa were oxidised in the fillets of rainbow trout (*Oncorhynchus mykiss*) during the storage time at -20°C. On the other hand, it has been confirmed that the rigor mortis phenomenon can alter fish muscle structure (Ando, Toyohara, Shimizu, & Sakaguchi, 1991). Moreover, the known post-mortem protein changes in fish muscle include the degradation of some large cytoskeletal proteins. Concerning the post-mortem time, different fish species have shown

different target proteins that correlate with texture properties. For instance, in trout 38 kDa protein was degraded, but in salmon and sea bass (*Dicentrarchuslabrax*) dystrophin are the important markers for texture properties (Bonnal et al., 2001). However, other proteins such as collagen, nebulin, tropomyosin, troponin and titin (connectin) are responsible for textural changes during the storage period. Thus, during the frozen storage partial dehydration of proteins as well as lipid-protein oxidation, and during post-mortem time autolytic enzyme activities followed by protein degradation, are the most important parameters which have negative effects on protein functionality (Leygonie, Britz, & Hoffman, 2012; Toyohara, Ando, & Shimizu, 1990).

Moreover, lipid and protein oxidation progress can be independent or in parallel, but mostly interactions happen between them (Zhang, Xiao, & Ahn, 2013). There are several types of chemical bonds which are responsible for this interaction in order to build units between lipid and protein molecules to create protein-lipid complexes:

1) Covalent bonds: An electron is shared between the protein and lipid molecules. Lipid double bonds and protein sulfhydryl groups can interact by this mechanism through the extra reaction of thiol to olefin (Robinson, 1966).
2) Hydrophobic interaction: This type of protein-lipid interaction only exist in the aqueous environment. Protein can interact with oil in aqueous system and it can alter the structure of protein. Because, the protein intramolecular hydrophobic bonds decrease and the unfolded protein can explain at the oil–water interface in the system.
3) Ionic binding: It is a coulomb attraction between charged groups with opposite charges. This type of interaction occur not only between the charged lipids and proteins but also with some groups which have inducible or permanent dipoles (-OH, -CO). As an example, charged arginine or lysine residues of protein can bond to the phosphate groups on the phospholipids through electrostatic forces.
4) Weak secondary forces: There are several types of weak bonds but the most important is the generation of Van Der Waals bonds. This bond exists on the non-polar ends of proteins and lipids in protein-lipid complexes.
5) Hydrogen bonding: This bond form between carbonyl groups of proteins and hydrogen of the hydroxyl groups of fatty acids, mono and diglyceride or the head groups of phospholipid like, phosphatidylserine or phosphatidylamine.

In general, lipid and protein oxidation is caused by the same set of factors. Also, with respect to the hydroperoxide and carbonyl generation, the kinetics of lipid and protein oxidation are similar but the variety of the protein oxidation products is more complex compared to lipid oxidation products due to more reactive targets in the proteins (Hematyar, Rustad, Sampels, & Kastrup Dalsgaard, 2019). The interactions between the oxidation products from lipid and protein have been reported in fish (Soyer & Hultin, 2000), chicken (Ayla Soyer, Özalp, Dalmış, &Bilgin, 2010), and beef (Estévez & Cava, 2004), which confirms that oxidation of lipid and protein start together and the processes interact with each other. Aalhus & Dugan (2014) have proposed that lipid oxidation is started earlier compared to protein oxidation. Due to the similar reactions in foods, the interaction between lipid and protein oxidation is boosted by the development of initial oxidative reactions (Hematyar et al., 2019). Saturated and unsaturated lipid aldehydes with covalent bonds can interact with amino acids, like tyrosine, aspartic acid, arginine, and methionine (Tyr, Asp, Arg, and Met) through a Schiff base formation or via Michael addition (Metz et al., 2004). The mentioned interactions result in increasing protein hydrophobicity and aggregation in fish muscle. Additionally, the development of lipid oxidation in fish muscle strongly associates with the formation of hemoglobin (metHb)

and myoglobin (metMb). Jonsson (2007) reported that the amount of thiobarbituric acid reactive substances (TBARS) and PV in herring muscle during frozen storage increased while the amount of haem-proteins decreased, indicating the possible relationship between lipid and protein oxidation products. Hematyar et al. (2019) reported several mechanisms and pathways of interaction between different products of lipid and protein oxidation and its final impact on fish muscle quality. Due to the interaction, several quality parameters, such as colour, flavour, protein hydrophobicity, aggregation, and tenderness are influenced.

Generally, the deterioration of fish muscle is faster than in other muscle foods; therefore, storage conditions play a key role to retain it. As fish is easily spoilt, it is important to keep the freshness of fish muscle until the consumption time. For that reason, several methods like chilling or freezing have been developed and used to keep the fish fillet freshness before the consumption. Frozen and refrigerated storage are common methods to keep the sensorial and nutritional values of fish muscle (Ortiz, Palma, González, & Aubourg, 2008; Sharifian, Alizadeh, Mortazavi, & Moghadam, 2014). Nielsen (2007) reported that in an ideal condition, fish muscle can be stored in the frozen condition (-20°C) for three months without significant alterations in the taste, colour, and firmness compared to the fresh fish. Hematyar, Masilko, Mraz, & Sampels (2018) reported even higher storage stability of common carp fillets during 6 months storage at -20°C. However, frozen storage can lead to some damages in fish muscle owing to ice crystal formation which can cause cell disruption (Kjaersgard, et al., 2006). In general, cell disruption influenced by the formation of ice crystal can enhance the lipid and protein oxidation during frozen storage. On the other hand, after slaughtering, chilling storage is widely used before technological processes or consumption to protect fish fillet quality. Sharifian et al. (2014) recommended maximum 9 days storage at +4°C as an ideal time for Grouper (*Epinepheluscoioides*) fillets with respect to the chemical, sensorial and microbial analysis. Although during refrigerated storage the enzymatic activity, spoilage microorganism's growth and the chemical reactions are slower, but still by elapsing the time they can lead to reduced fish muscle quality (Cakli, Kilinc, Cadun, Dincer, & Tolasa, 2007). Furthermore, during chilling conditions post-mortem ageing occurs, being able to influence the quality of fish fillets. It is well known that rigor mortis is associated with oxidation development, protein and adenosine triphosphate (ATP) degradation, and also pH reduction (Fu et al., 2014). Suarez, Abad, Ruiz-Cara, Estrada, & Garcia-Gallego (2005) reported a significant correlation between sea bream fillet firmness and collagen alteration. In the period of rigor mortis, collagen is responsible for the softening of fish flesh. Post-mortem changes during refrigerated (+4°C) or ice storage (0°C) can be monitored in order to find the role of autolytic activity and protein degradation on fish fillet quality. Hematyar, et al. (2019) mentioned that the lipid oxidation products can promote protein oxidation in fish fillets, particularly during frozen storage, while in the period of post-mortem ageing protein oxidation dominates. Therefore, investigation on fish flesh quality during storage time (freezing and chilling) can provide some information about the mechanisms, products, and targets of lipid-protein oxidation.

In this study, we investigated some chemical and physical methods for evaluating the fish muscle quality during long and short-term storages. The possible mechanisms and pathways of lipid-protein interactions and their influence on the final flesh quality were also assessed. The evaluation of fish muscle quality was done in relation to the progress of lipid and protein oxidation, fatty acid composition, colour, textural parameters, and proteomic analyses. We considered TBARS and PV as markers of lipid oxidation and carbonyls respectively which indicate protein oxidation progress. In addition, firmness and liquid loss were analysed as indicators for fish muscle quality. Moreover, we examined the progress of rigor mortis and its effect on pH and microbial spoilage during post-mortem time. Furthermore, we performed proteomic analyses on fish muscle in order to find a protein profile as well as monitor the

protein oxidation development. The combination of the measured parameters gives a better overview and understanding of the involved mechanisms in oxidation and its impact on fish muscle during the storage time.

THESIS AIM AND SPECIFIC OBJECTIVES

The purpose of the current thesis was to study the effects of storage condition on the development of lipid and protein oxidation and to monitor the mechanisms and pathways of oxidation as well as the correlation between lipid-proteins in fresh and stored fish fillets. Also, the impact of oxidation on some quality parameters was investigated. We analysed lipid oxidation with respect to TBARS and PV, protein oxidation, protein profile and peptide identification with 2,4-DiNitroPhenyl Hydrazine, 1D, western blot, native gel and MALDI-TOF/MS/MS methods respectively. Additionally, lipid class composition, fatty acid, colour, firmness, WHC, pH, rigor mortem, biogenic amines were assessed by TLC, gas chromatographic, Minolta, textural analyser, pH meter, rigor board and UPLC instruments respectively.

Specific objectives:

1- Examine the amount of TBARS and carbonyls as indicators of lipid and protein oxidation progress during short and long term storage in muscle of two fish species;
2- Examine the percentage of fatty acids and lipid class composition in common carp (*Cyprinus carpio* L.) and perch (*Perca fluviatilis* L.) for monitoring the impact of fat content on the development of lipid oxidation;
3- Examine the effects of oxidation progress on the textural parameters of fish muscle;
4- Examine the influence of different rearing systems (RAS and pond) on the flesh quality of perch (*Perca fluviatilis* L.) during storage time;
5- Examine the effects of rearing systems on the rigor onset, pH and progress of biogenic amines during refrigerator storage;
6- Examine the proteome changes of Eurasian perch (*Perca fluviatilis* L.) fillet from two different rearing systems influenced by frozen storage.

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CHAPTER 2

RELATIONSHIP BETWEEN LIPID AND PROTEIN OXIDATION IN FISH. A REVIEW

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REVIEW ARTICLE

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Relationship between lipid and protein oxidation in fish

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Abstract

Reactive oxygen species (ROS) are generated in all aerobic organisms. Free radicals are highly reactive ROS that cause damage to biological materials. Fish is rich in polyunsaturated fatty acids, and hence, very prone to lipid peroxidation. Both lipid and protein oxidations are important for quality loss during storage of fish, with high impact on taste and texture. Also, there are interactions between protein and secondary lipid oxidation products (aldehydes) that occur in foods because the oxidation products from one reaction can further react with both lipids and proteins respectively. This review focuses on the mechanisms and pathways of the lipid and protein oxidation and their possible relationship. Additionally, the target amino acids and final impacts of this relationship were considered. We propose that the products of lipid oxidation promote protein oxidation in fish rather than the other way around specially, during frozen storage, while during postmortem changes protein oxidation dominates. Finally, it seems that, secondary products of lipid oxidation might have more impact on the functionality of proteins from both Michael addition and Schiff base reaction rather than lipid hydroperoxides and lipid radical transfer.

KEYWORDS
fish, free radicals, interaction, Lipid oxidation, protein oxidation

1 | INTRODUCTION

Oxidation in food is a process that concerns both lipids and proteins by the effect of reactive oxygen species (ROS) (Bernardini et al., 2011). Oxidative modifications can cause numerous functional consequences and lead to changes in food texture, water holding capacity (WHC), digestibility and juiciness in fish meat (Baron, Kjaersgard, Jessen, & Jacobsen, 2007). Also, Sarma, Reddy, and Srikar (2000) found a negative correlation between lipid oxidation parameters and functional properties of the protein in Indian oil sardine, indicating their interdependence. However, still little is known about the kinetics of protein oxidation, the interaction of protein and lipid oxidation and the subsequent impact on muscle food quality (Baron et al., 2007; Kjaersgard & Jessen, 2004).

For many years, the focus of food was on lipid oxidation, not only because of its effect on taste and shelf life, but also because the lipid oxidation products can more easily be measured (hydroperoxides, hexanal and MDA) than protein oxidation product and therefore have been detected and correlated to off-flavour in food. Especially in fish, which is rich in *n*-3 polyunsaturated fatty acids (PUFA), there is a high risk of quality loss due to oxidation (Jeremiah, 2001; Medina, Gallardo, & Aubourg, 2009). Besides lipid oxidation, the oxidation of proteins can also cause quality changes in the fish fillet. In general, the same factors causing lipid oxidation will also cause protein

oxidation. The kinetics of protein and lipid oxidation with respect to the generation of hydroperoxide and carbonyls are quite similar but the diversity of the protein oxidation products are more complex than those of lipid oxidation due to more reactive targets in the proteins. The progress of initial oxidative reactions in food is enhanced by the interactions between the proteins and lipids due to the similarity of the oxidation reactions. The development of lipid and protein oxidation can occur in parallel or independently, but often there are interactions between them (Zhang, Xiao, & Ahn, 2013). Several authors reported a good correlation between oxidised products of lipid and protein in fish (Soyer & Hultin, 2000) beef (Estévez & Cava, 2004) and chicken (Soyer, Özalp, Dalmış, & Bilgin, 2010), which indicates that protein and lipid oxidation start together and can interact with each other. In contrast to those conclusions, Aalhus and Dugan (2014) have suggested that the products of lipid oxidation can promote protein oxidation since lipid oxidation may start earlier.

In the initiation of lipid oxidation, products can react with proteins and vice versa, but the most well-described reaction is the reaction between secondary lipid oxidation products and primary amino groups on proteins (Schwenke, 1978; Burcham, & Kuhan, 1996).

As touched upon above, the reaction between secondary lipid oxidation products and amino acids also takes place in fish. Saturated lipid aldehydes become covalently bound to susceptible and functional groups of proteins like N-terminal groups of Tyr, Asp, Arg and Met (Metz et al., 2004), the ε-NH2 group of Lys and Cys-SH (Metz et al., 2004) through a Schiff base formation while unsaturated aldehydes also can react via Michael addition (Cai, Bhatnagar, & Pierce, 2009). By these interactions proteins hydrophobicity and aggregation are increased in fish flesh. In addition, the progress of lipid oxidation in fish muscle strongly correlates to the formation of metHb/metMb and also leads to the reduction in extractability haem proteins. For example, during frozen storage of herring fillets, peroxide value (PV) and thiobarbituric acid reactive substances were increased while the amount of haem-proteins decreased (Jonsson et al., 2007).

This review aims to give an overview of the lipid and protein oxidation, the known pathways and correlations between lipid and protein oxidation in raw and postmortem conditions and also the effects of oxidation with a special focus on fish fillet quality.

2 | MECHANISMS OF LIPID AND PROTEIN OXIDATION

In order to investigate lipid–protein correlations in fish fillet, the mechanisms of lipid and protein oxidation should be understood in depth. Particularly, the control of possible catalysts has a key role because free radical chain reactions can be swiftly boosted by catalysts.

Lipid oxidation is a key factor, leading to a decline in food quality, predominantly of those food products, which contain high amounts of unsaturated fatty acids (Secci & Parisi, 2016). Generally, the potential initiators of lipid oxidation can also initiate the protein oxidation (Xiong, 2000). However, the mechanisms, pathways and also the products of protein oxidation are different (Stadtman, 2006). The functional groups, which are located in the amino acid residues side chain and the peptide backbone are the targets for ROS.

The effect of lipid oxidation in muscle food (Estévez & Cava, 2004) and seafood (Secci & Parisi, 2016; Mariutti, & Bragagnolo, 2017) and protein oxidation (Estévez, 2015; Soladoye, Juarez, Aalhus, Shand, & Estevez, 2015) has been reviewed extensively whereas correlation between the lipid and protein oxidation has not really been touched upon in a review.

2.1 | Lipid oxidation

Unpleasant off-flavours and formation of volatiles can develop due to the oxidation of PUFA. The most important factors which can affect rancidity in fish muscle are the high content of PUFA and also the presence of pro-oxidants, especially the parts containing haem groups (Richard, & Hultin, 2002).

Autoxidation enzymatic catalysed oxidation and photosensitized oxidation are three major mechanisms in lipid oxidation.

Autoxidation in meat and fish can be initiated by light, heat and the presence of metal ions and radicals (Sampels, 2013). Autoxidation leads to the formation of the primary oxidation products, hydroperoxides (ROOH). Once the oxidation process has started, a cascade of reactions will occur with each new molecule increasing the reaction speed and variability (Heinonen, Meyer, & Frankel, 1998). ROOH from lipid oxidation decompose easily at a high temperature or in the presence of metals to secondary products such as aldehydes, short chain hydrocarbons, alcohols, esters, acids and ketones (Choe & Min, 2006). Finally, termination products can be crosslinking products, for example, where two radicals react with each other, thereby terminating the chain reaction caused by radical reaction.

The atmospheric triplet oxygen is the most common oxygen species involved in the oxidation of lipids but in the photosensitized reaction, both singlet and triple oxygen are two types of oxygen having more interactions with lipids (Foote, 1976), Singlet oxygen has been suggested to react 1,450 times faster with linoleic acids than triplet oxygen (Rawls, & Santen, 1970). Singlet oxygen is needed to start the so called type II photosensitized oxidation process, which can react with unsaturated lipids (Foote, 1976), but compared with type I reaction the type II reaction generate different types of hydroperoxide products. The greater importance is the energy of oxygen, which has a significant impact on the initiation of the oxidation reaction.

Metal catalysis can be considered as an important lipid oxidation reaction in fish muscle, this means that after post-slaughter processes, the released haem iron (ferrous (+2)) is converted to ferric (+3) and starts the autoxidation progress. Both haem proteins (haemoglobin [Hb] and myoglobin) can increase the lipid oxidation in fish fillet and other muscle foods (Kanner, 1994). Richards and Hultin (2002) reported that the blood residue in fish fillet, catalyses lipid oxidation during storage of fatty fish and bleeding was also shown to retard lipid oxidation of minced trout muscle during storage at +2°C. Maqsood and Benjakul (2011) showed that the initiation and propagation of lipid oxidation in the un-bled samples compared with the bled samples were more marked.

In addition, there are several enzymes in fish, which are capable of catalysing lipid oxidation such as, lipoxygenases and myeloperoxidases. The first one exists in fish skin and gills and can catalyse the incorporation O_2 into an unsaturated fatty acid and generate ROOH, and the second one initiates lipid oxidation in the presence of halides and hydrogen peroxide. During the process, this can be critical because the lipid, oxygen and blood interaction will be increased (Mozuraityte, Kristinova, Rustad, & Storro, 2016).

2.2 | Protein oxidation

Foods are constantly exposed to ROS and this will not only cause lipid oxidation but also protein oxidation. However, for several decades proteins were ignored as a target for ROS, in the opposite of lipid oxidation, which was investigated very deeply. In contrast to lipids, proteins are complex macromolecules arranged in 3D-structures, and when oxidation occurs it leads to various changes in the proteins, both chemical changes on individual amino acids (Davies, Delsignore, & Lin, 1987) such as Met (Dalsgaard et al., 2010) Tyr (Dalsgaard, Nielsen, Brown, Stadler, & Davies, 2011) His and Trp (Dalsgaard, Nielsen, & Larsen, 2007) Lys and Arg (Dalsgaard, Otzen, Nielsen, & Larsen, 2007; Lund, Heinonen, Baron, & Estevez, 2011) but also structural changes may occur (Davies & Delsignore, 1987). Because the functions of protein are very specific, oxidative modifications can cause numerous functional consequences and lead to changes in food texture, WHC, digestibility and juiciness (Baron et al., 2007; Sarma et al., 2000). Protein oxidation may be caused directly by ROS and reactive nitrogen species or indirectly as the result of reactions with products from lipid oxidation with reducing sugars or carbohydrate (Lund et al., 2011). On the other hand, nucleophilic reaction on the carbonyl groups of free sugars and aldehyde on the side chain of amino acids can lead to production of Schiff base products (Dalsgaard, Nielsen, & Larsen, 2006, Dalsgaard, Otzen et al., 2007). Generally, the pathways of protein carbonylation can be divided to direct oxidation, metal-catalysed oxidation, reaction with free sugars and also lipid peroxidation products (Michael adducts) (Fedorova, Bollineni, & Hoffmann, 2014). Due to side chain oxidation of some amino acids (Arg, Lys, His and Pro) or backbone oxidation of Asp, Pro and Glu residues carbonylation can be considered as a permanent and destructive (Hawkins & Davies, 2001) indicator of protein oxidation (Nystrom, 2005).

Protein oxidation is initiated when a hydrogen atom is abstracted from the protein to generate a 0140 carbon-centred radical (C·) and in the presence of oxygen is converted to an alkylperoxy radical (COO·). The following reaction of (COO·) with hydrogen atom abstraction from another molecule leads to alkyl peroxide (COOH) formation. Subsequent reactions lead to the formation of the alkoxy radical (CO·) and hydroxyl compounds (COH). In addition, two carbon-centred radicals (alkyl-radical such as ethane, methane and propane) can react with each other in the absence of oxygen to generate carbon–carbon cross-linked derivatives (Papuc, Goran, Predescu, & Nicorescu, 2017). The termination reaction, which in relation to fish and meat quality may affect tenderness (Soladoye et al., 2015).

Additionally, aromatic amino acids like Tyr, Trp, His and Phe are very susceptible to oxidation (Hawkins & Davies, 2001). During oxidation of aromatic amino acids, phenoxyl radicals will be formed from tyrosine and their metabolites, dityrosine and other products, are generated. This occurs especially when tyrosine is close to tyrosol radicals and also tyrosyl radicals which cannot be repaired because they are not reductants (Aeschbach, Amadoò, & Neukom, 1976).

On the other hand, the amino acids, which are aliphatic but do not contain sulphur like Pro or Arg are oxidized via another way. In this group, oxidation takes place by hydrogen abstraction at the α-carbon generating a carbon centred radical (Stadtman, 1993). This reaction occurs at the terminal amine of the Lys side-chain and sites distant from deactivate α-amino group. The generated product will be different depending on the presence or absence of oxygen (Stadtman, 1993).

Protein oxidation via metal-catalysed cleavages is the main reason of oxidative damage in vivo systems like fish (Moller, Rogowska-Wrzesinska, & Rao, 2011) and also in the postmortem fish muscle.

In some proteins, metal ion-catalysed oxidation systems can easily oxidize the side-chains of amino acid residues. Lys, Pro and Arg are the most important targets of metal catalysed oxidation (Adolfo Amici, Tsai, & Stadtman, 1989). Requena, Chao, Levine, and Stadtman, (2001) reported glutamic and then aminoadipic semialdehydes are respectively very important carbonyl products in metal catalysed oxidation systems. For example, Lys can be a target for Fe (II)-catalysed oxidation system. In the mentioned mechanism, the chelate form of Fe (II) and amino group of Lys can generate a hydroxyl radical by a reaction between Lys and hydrogen peroxide. Hydroxyl radical preferentially attacks to the Lys moiety to convert Lys to a 2-amino-adipic-semialdehyde residue. The similar reaction with other amino acids by Fe (II) can produce carbonyl derivatives. The site-specific mechanism is supported by the confirmation that the metal-catalysed reactions are prevented by catalase but not by ·OH scavengers, maybe because the scavengers are not be able to compete with the "caged" reaction at the metal binding site between amino acids and ·OH.

Amadori products, which have been generated via Schiff base products rearrangement are highly susceptible to the degradation and metal-catalysed oxidation reaction. Consequences of the reactions lead to protein cross-linked adducts and Maillard reaction products (Lund et al., 2011).

The major mechanisms in protein oxidation are still unclear because there are only a few methods available to evaluate protein oxidation mechanisms, but the number of reaction products is large. Detection of carbonyl groups, the formation of dityrosine and changes in sulphydryl groups are the most common methods to detect and quantify protein oxidation. In addition, there are some advanced methods such as fluorescence spectroscopy, and electron spin resonance, immune-spin trapping in combination with mass spectrometry used to investigate the mechanism of protein oxidation (Dalsgaard et al., 2014).

2.2.1 | Function of proteins as antioxidants

On the other hand, in some cases, proteins besides being the target for oxidation are also known to be able to function as antioxidants. The mechanisms of proteins and amino acids as antioxidants in food have been related to their ability to chelate pro-oxidative metals; sulphydryl groups, which exist in the proteins and amino acids can inactivate free radicals (Viljanen, Kylli, Hubbermann, Schwarz, & Heinonen, 2005). In some cases, the proteins may act as a shield between ROS and the lipid (Dalsgaard, Sorensen et al., 2011). Carnosine can prevent lipid oxidation in various model systems by free radical scavenging or metal ion chelating (Liu, Xing, Fu, Zhou, & Zhang, 2016). His residues and carnosine can postpone oxidation by removing metal ions from the surface of other macromolecules (Guiotto, Calderan, Ruzza, & Borin, 2005). Furthermore, some peptides, which contain Trp or Tyr at the C-terminus have shown to possess a high radical scavenging ability (Saito et al., 2003) and as mentioned above, for example, dityrosine formation as a consequence of two tyrosine radicals reacting with each other will terminate the radical (Dalsgaard, Sorensen et al., 2011). The amount of riboflavin, which is responsible for photo-oxidation is variable in different fish species (Brjekkan, 1959), may play an important role as well. It seems that fish with higher amount of proteins contain more riboflavin. Therefore, Trp and Tyr can be considered as a main target of photo-oxidation in fish fillet, which are the only amino acids that can compete with oxygen or unsaturated fatty acids in the quenching of triplet state riboflavin (Cardoso, Franco, Olsen, Andersen, & Skibsted, 2004; Dalsgaard, Sorensen et al., 2011). However, the sequence and categories of amino acid might have a key role in the peptides antioxidant activity (Liu et al., 2016). Imidazole ring in the R group of His has the ability of metal ion-chelating, lipid peroxyl radical trapping and hydrogen donating (Chan & Decker, 1994). In addition, Pro-His-His sequence showed higher antioxidant ability in the linoleic acid system compared with other synthetic peptides (Liu et al., 2016), while, the mechanism of hydrophobic amino acids such as Trp and Try might differ. The presence of peptides is enhanced at the water–lipid interface which can approach to the lipid phase and scavenge free radicals (Ranathunga, Rajapakse, & Kim, 2006).

Hence lipid oxidation in fish could also be retarded due to the simultaneous presence of certain proteins and amino acids. However, this connection is also still widely unexplored.

3 | IMPACT OF LIPID AND PROTEIN OXIDATION ON FISH FILLET TRAITS

3.1 | Lipid oxidation and quality parameters

Sensory, nutritional value and colour are the major quality characteristics, which can be affected in muscle foods by lipid oxidation. Several authors reported the negative effects of lipid oxidation on the sensory aspect of fish fillet (Baron et al., 2007; Yin, Luo, Fan, Wu, & Feng, 2014). Yin et al. (2014) studied effects of frozen storage on grass carp (*Ctenopharyngodon idellus*) fillet and reported that the sensory parameters (colour, odour, morphology and muscle elasticity) decreased significantly d by the effect of lipid oxidation.

Estévez, Ventanas, and Cava, (2005) reported a significant correlation between lipid oxidation and fat content in liver of pâté. Fat content might have a key role in the development of lipid and protein oxidation in the muscle foods (Estevez, Morcuende, Ventanas, & Cava, 2003; Stadtman, 1990). In order to find a correlation between fat content and the development of lipid oxidation we compared PV results of two different kinds of fish species (lean and fatty fish). Comparing the studies by Saeed and Howell (2002) and Baron et al. (2007) on Atlantic mackerel (*Scomber scombrus*) rainbow trout (*Oncorhynchus mykiss*), respectively, reveals that in fatty fish lipid oxidation started earlier compared with lean fish and PV values were higher during storage at −20°C in fatty fish.

In most fish, the major contributor to the colour of muscle is myoglobin. During storage, ferrous oxymyoglobin (Fe^{2+}) oxidized to ferric metmyoglobin (Fe^{3+}), which is responsible for a discoloration reaction in fish fillets (Papuc et al., 2017). Secondary lipid oxidation products (aldehydes) can, furthermore, alter the myoglobin structure via covalent bonds and change the fillet colour during the storage time (Lynch & Faustman, 2000). In addition, brown pigments in fish can be generated via lipid–protein interaction. In this case, lipid peroxide can interact with active types of proteins and lead to the transformation of the light coloured or colourless precursor to brown pigments (Hidalgo & Zamora, 2000).

3.2 | Consequences of protein oxidation

The hydrophobicity, solubility, WHC tenderness and texture are the most important quality parameters, which depend directly on protein oxidation in fish fillet (Lund et al., 2011). Oxidation in a side chain of amino acids can produce carbonyl groups that eventually result in a loss of solubility and protein aggregation (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004).

3.2.1 | Hydrophobicity

Hydrophobic residues, in the native form of proteins, are hidden. In a semi oxidized protein, the alteration of secondary and tertiary of protein structure exposes the hydrophobic residues to the proteases enzyme substrates followed by protein degradation (Jung, Hohn, & Grune, 2014), while heavy oxidation resulted in protein aggregation via cross-linked proteins (Hohn et al., 2011). Aggregated proteins are stable against enzymatic degradation (Reeg & Grune, 2015).

3.2.2 | Water holding capacity

Protein oxidation has a negative effect on the WHC. As the accessibility of the polar groups to pro-oxidants, which are present in fish muscle, is very high, they are more prone to oxidative reactions (Standal et al., 2018). Protein carbonylation leads to the loss of amino groups, which in turn results in the alteration of the distribution of the

electrical charges and the overall arrangement of myofibril protein. It appears that one result of intensive oxidative protein modification is a change in the isoelectric points of proteins. Therefore, the oppositely charged groups are more attracted to each other and thereby decrease the amount of water that is held by the protein. Moreover, the repulsion of the myofibril protein structures is reduced due to the isoelectric point therefore the protein structures can be more compact and decrease WHC (Huff-Lonergan & Lonergan, 2005).

3.2.3 | Tenderness and firmness

The tenderness of fish fillets can change as a result of protein oxidation. There are two hypotheses for a decrease in tenderness in relation to protein oxidation:

a Amplification of the myofibrillar structure through the formation of MP cross-linking (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010) particularly, in the presence of oxygen. Myosin proteins especially, MHC is susceptible to oxidation due to the presence of Cys in the root tail of myosin, which is responsible for protein cross-link. The results of this cross-link may decrease the fish muscle tenderness. Glutathionylation of particular Cys in myosin binding protein C and troponin I might be responsible to change the sensitivity of myofilament to calcium (Patel, Wilder, & Solaro, 2013). In several studies, myosin has been reported as a target of glutathionylation and also protein oxidation (Passarelli et al., 2010) but still there is a lack of knowledge about the functional and structural alteration in myosin by the effect of specific redox sites.
b μ-Calpain inactivation (Rowe et al., 2004). For peptide bonds hydrolysis by calpains, electron transfer is needed on the active side chain of His and Cys residues. In this case, carbonyl derivatives of some amino acids, such as His, lead to the formation of inter and/or intra-protein disulphide cross-links (Martinaud et al., 1997). Because SH-containing Cys and His residues are present at the active sites of both μ-calpain and m-calpain enzymes and these products might be inactivated by oxidation and lead to reduce fish muscle tenderness (Carlin, Huff-Lonergan, Rowe, & Lonergan, 2006).

On the other hand, it has been proved that due to protein oxidation, firmness is reduced during frozen storage by the impact of ice crystal formation and cell disruption (Hematyar, Masilko, Mraz, & Sampels, 2018; Subbaiah et al., 2015).

It seems that myoglobin and myofibril are the most important proteins to impact on fish quality. Probably, the formation of covalent bonds and cross-links, by the impact of lipids and proteins oxidation are responsible for quality changes in the fish fillet. Therefore, extensive oxidation in fish muscle often leads to higher firmness while moderate protein oxidation can decline the firmness due to more unfolded protein structures. Additionally, degradation and aggregation of proteins in the postmortem fish muscle lead to higher firmness and lower tenderness. On the other hand, during frozen storage formation of unfolded proteins due to oxidation or lipid–protein interaction probably are responsible for reduction in firmness.

4 | CORRELATION AND INTERACTION OF LIPID AND PROTEIN OXIDATION

During the storage of fish fillet, proteins are exposed to oxidized lipids or secondary breakdown products that may cause some changes in protein functionality including insolubilization, polymerization, loss of enzymatic activity and formation of lipid–protein complexes (Howell, Herman, & Li-Chan, 2001).

4.1 | Mechanisms of interaction

Two mechanisms can be considered for the interaction of lipids-proteins:

a First, hydroperoxide decomposes to secondary lipid oxidation products, for example, malonaldehyde that may react further with amino groups (Schaich & K. M., 1976) or for unsaturated lipid aldehydes reacting with Cys, His, or Lys through Michael addition (Stadtman & Levine, 2000).
b Some products of lipid oxidation (hydroperoxides, lipid free radicals and volatile secondary oxidation products) can react with proteins to generate protein-centred free radicals (Saeed, Fawthrop, & Howell, 1999).

Secondary lipid oxidation products can bind to proteins in two main ways by either binding to an active site or cavity or at less well defined hydrophobic patches at positions close to the surface of the protein (Fillery-Travis, Mills, & Wilde, 2000). Aldehydes as a product of metal catalysed lipid oxidation cause, protein–lipid aggregation via crosslink bonds (Gardner, 1979). The pathways for the interaction of lipid oxidation products (carbonyls) with proteins are formation of Schiff base and Michael-type (Refsgaard, Tsai, & Stadtman, 2000). In the carbonyl amine reaction, aldehydes can bind directly to amino groups in proteins through covalent bonds. Interaction of dimethylamine and formaldehyde from trimethylamine-N-oxide may result in decreased WHC as formaldehyde may form crosslinks between proteins, which have been shown to decrease WHC and protein solubility in the Gadiform Fish fillet (Nielsen & Jorgensen, 2004).

Also, the products of lipid oxidation might have interaction with proteins on the hydrophobic groups site, which lead to generate lipid-soluble fluorescence products. The consequence of this interaction in the defatted samples can be resulted to decline in soluble protein hydrophobicity (Liang, 1999) upon when the secondary and tertiary structure of proteins are changed (Meng, Chan, Rousseau, & Li-Chan, 2005). This reaction mostly happens in the presence of some prone amino acids such as His, Lys, Pro, Arg, Tyr, Trp, Cys and Met via side chain reaction with lipid oxidation products. As soon as protein unfolding occurs at the water–lipid interface, the hydrophobic groups are absorbed and interact with the lipid phase. While, the negatively charged groups of protein can remain in contact with water molecules and hence increase the solubility or reduce the risk of protein-protein aggregation (Gitlin, Carbeck, & Whitesides, 2006; Kramer, Shende, Motl, Pace, & Scholtz, 2012).

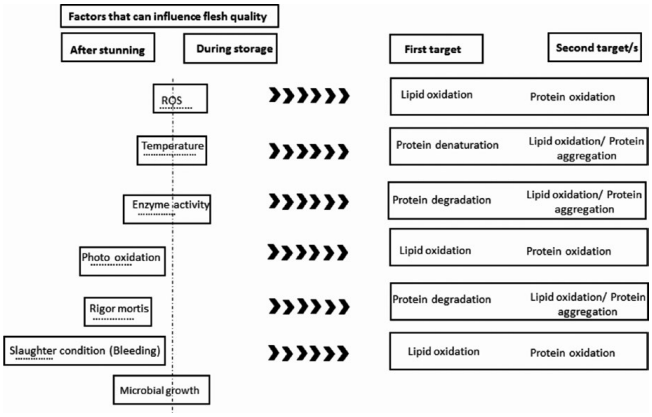

FIGURE 1 Main factors that can influence fish flesh quality before and after storage time and prefer primary and secondary targets. The efficiency of parameters which affect on fish flesh quality in the time period is indicated by dotted lines

Thus, it appears that the covalent bonds are a very important type of bonds between free amino groups of Cys, His and Lys with MDA via side chain pathway (Pizzimenti et al., 2013). For a better understanding of protein–lipid interaction, it is necessary to know more about the water–lipid bonds and also the lipid–protein relationship, as it seems that there is a close relationship between them (Alzagtat & Alli, 2002). In the frozen fish muscle due to the absence of water molecules, lipid–protein or protein–protein interactions are dominated. Additionally, the interactions depend on the secondary products or radicals predominate that can be specified by which proteins get involved in the lipid oxidation reaction chain (Ladikos & Lougovois, 1990). Furthermore, the stability of the proteins and volatiles may increase via non-covalent bonds during the storage time of muscle foods.

4.2 | Role of haemoglobin and myoglobin on the progress of lipid oxidation

It has also been demonstrated in model systems that lipid oxidation can increase in the presence of protein radicals (Østdal, Davies, & Andersen, 2002). A good example for this kind of interaction in fish muscle has been reported by Richard and Hultin (2002) that revealed oxidized haemoglobin (deoxyhemoglobin) is a powerful catalyst of lipid oxidation.

Haemoglobin (Hb) autoxidation rate in different fish species, might be influenced by their residue (Powers, 1972). Richards and Hultin (2003) revealed that Hb from mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) showed more pro-oxidative activity than from trout (*Onchorhynchus mykiss*) Hb. This would be related to the frequent migration, which made Hb more susceptible to autoxidation. Probably, mackerel blood contains more lipid oxidation promoters or less powerful inhibitors (Richards & Hultin, 2003). However, Maqsood and Benjakul (2011); Maqsood, Singh, Samoon, and Munir (2011) reported that active migratory fish like seabass (*Lates calcarifer*) had less pro-oxidative Hb. In addition, Hb autoxidation in the fish from cold water was 10-fold faster than warm water fish (Maqsood & Benjakul, 2011). His residue may be situated away from the centre making Hb more resistant to oxidation (Jensen, 2001). Probably, when His and Phe residues are located on the distal part of the haem group the accessibility of iron to the mentioned amino acids is decreased that leads to less interaction between Hbs and lipid oxidation products. The rate of lipid oxidation can be affected by different Hbs formations in fish flesh (Maqsood, Benjakul, & Kamal-Eldin, 2012). Richards and Hultin (2002) reported that deoxyHb is a stronger catalyst for lipid oxidation compared with oxyHb. Formation of deoxyHb releases the iron from the inside of the porphyrin group, which is a catalyst for lipid oxidation and lipid–protein interaction. Immediate bleeding would keep the freshness of muscle for a longer time and maintenance the fillet firmness during the storage time. It has been demonstrated that inadequately bled or non-bled fish shows lower overall quality in the fish fillet as bleeding decreases the total haemoglobin in the muscle (Richards & Hultin, 2002).

In fish, myoglobin oxidation and lipid oxidation are associated and influence each other (Chaijan, 2008). Secondary lipid oxidation products (aldehydes) can modify the stability of myoglobin and generate adducts through a covalent modification with myoglobin but in the absence of lipid oxidation products metal catalyse via side chain reaction can unfold the proteins. In line with this, Lynch and Faustman (2000) proposed that pro-oxidant activity of metmyoglobin and oxymyoglobin oxidation will be increased by aldehydes. Furthermore, metmyoglobin and H_2O_2, resulting from oxymyoglobin oxidation, can provoke lipid oxidation. In one way the products of oxymyoglobin (metmyoglobin and H_2O_2) are necessary to start lipid oxidation and on the other way aldehydes can change myoglobin stability and promote oxymyoglobin oxidation.

4.3 | Myosin–lipid interaction

Myosin is another protein in fish that has a weak tendency to form a complex with lipids, but the form of myosin can change during the storage due to unfolding. However, linoleic acid hydroperoxides are highly

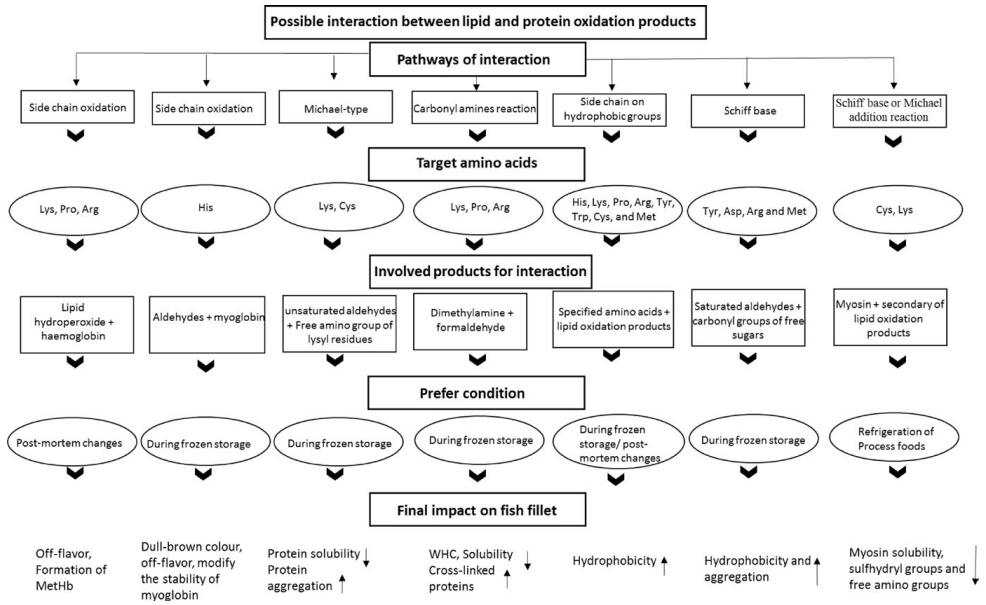

FIGURE 2 The main possible interaction pathways with the target amino acids and involved products of lipid and protein oxidation with respect to the ideal condition. Main impact of the mentioned interaction on the final flesh quality

destructive for myofibrillar structures that can precipitate and denature the A-band (predominantly myosin). Chopin, Kone, and Serot (2007) investigated the interaction between fish myosin and secondary of lipid oxidation products and found a correlation between myosin solution and aldehydes, which leads to a decline of protein solubility, due to aggregations, also supported by (Buttkus, 1966). It seems that the interaction is taking place at some uncovered and new opened sites of unfolded myosin chain. During this interaction myosin solubility, sulphydryl groups and free amino groups significantly decreased, thus supporting the idea of Schiff base and/or Michael addition reaction taking place.

In order to make more visual, we showed some mechanisms and target proteins in the relationship between lipid and protein oxidation products in a schematically graph (Figures 1 and 2).

During postmortem of fish muscle the lack of ATP and anaerobic conditions leading to the antioxidants consumption which are resulted to the oxidation development. Probably during postmortem changes, first metal catalysed oxidation in proteins are dominated followed by lipid oxidation development. We would say maybe protein oxidation started earlier than lipid oxidation or both are in parallel. Therefore, bleeding or washing the muscle after stunning can be considered as a main factor to reduce this interaction. On the other hand, it appears that during frozen storage, formation of secondary products of lipid oxidation has a key role in the lipid–protein interaction via Schiff base reaction in the side chain of amino acids.

Secondary lipid oxidation products might have more impact on the functionality of proteins from both Michael addition and Schiff base reaction rather than lipid hydroperoxides.

5 | CONCLUSION

The present review summarizes the main actions of protein oxidation and the possible connection to lipid oxidation with a focus on fish quality. Until now, both oxidation processes have been investigated more or less separate from each other. However, both processes occur in parallel in fish and fish products. The reaction products can then react further with each other and form either volatile or non-volatile stable products. Formation of 'induced' protein–lipid complexes is the result of this interaction.

We propose that the products of lipid oxidation promote protein oxidation in fish rather than the other way around specially, during frozen storage. While during postmortem changes, protein oxidation is dominating. This hypothesis needs to be explored in model systems as well as on real fish samples. In addition, a comparison of lean and fatty fish should be made. There is the possibility, that retarding lipid oxidation could also slow down protein oxidation and hence increase shelf life of fish. Furthermore, there is a strong need to investigate how oxidation products influence the kinetics of the ongoing lipid and protein oxidation processes to better prevent oxidation and food spoilage.

Finally, it seems that, secondary products of lipid oxidation might have more impact on the functionality of proteins from both Michael addition and Schiff base reaction rather than lipid hydroperoxides.

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CHAPTER 3

NUTRITIONAL QUALITY, OXIDATION, AND SENSORY PARAMETERS IN FILLETS OF COMMON CARP (*CYPRINUS CARPIO* L.) INFLUENCED BY FROZEN STORAGE (–20 °C)

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Nutritional quality, oxidation, and sensory parameters in fillets of common carp (*Cyprinus carpio* L.) influenced by frozen storage (−20 °C)

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Abstract

The aim of this study was to find the effects of frozen storage on lipid and protein oxidation, firmness, liquid loss, sensory properties, and nutritional values in common carp (*Cyprinus carpio*) fillets during 6 months of frozen storage (−20 °C). Thiobarbituric acid-reactive substances, peroxide value, and carbonyl concentration were significantly increased after the 4th, 2nd, and 8th weeks, respectively. The firmness of fillets decreased, whereas the liquid loss increased. In contrast, sensory evaluation did not show any significant changes. The amount of monoacylglycerols and diacylglycerols decreased significantly after 8 weeks. The L^* and b^* values increased significantly after the 16th and 3rd weeks but a^* showed a minor increase. The value of pH increased significantly until the 4th week. The results indicate that the development of lipid and protein oxidation was not intense in the period of 24 weeks of frozen storage, and the fish was in an acceptable condition.

Practical applications

The demand of consumers for carp is increasing owing to the high content of essential polyunsaturated fatty acids. However, deterioration of fish fillets during the storage time can be a major problem as it leads to a loss of market acceptability. Therefore, monitoring the changes in lipids, proteins, sensory aspects, nutritional quality, and firmness during frozen storage is important. This study showed that after 24 weeks of storage at −20 °C, the products of lipid and protein oxidation increased but all the measured quality parameters were still within acceptable values.

1 | INTRODUCTION

Fish has an excellent nutritional value, providing high amounts of polyunsaturated (PU) *n*−3 fatty acids (FAs) and high-quality protein. Carp is an important farmed fish species in the world owing to its easy cultivation, fast growth rate, and high feed efficiency ratio (Tokur, Ozkutuk, Atici, Ozyurt, & Ozyurt, 2006). In central European countries, carp is traditionally consumed during the Christmas season (Zajíc, Mráz, Sampels, & Pickova, 2013).

Lipid oxidation is an important problem, leading to a decrease in food quality, particularly of those food products that contain large amounts of unsaturated FAs (Secci & Parisi, 2016). Lipid oxidation in foods, such as fish particularly containing polyunsaturated fatty acids (PUFAs), has been demonstrated to be connected to the decrease of the quality of the flesh in the period of storage, development of off-flavor components, and loss of nutritional value (Maqsood, Benjakul, & Kamal-Eldin, 2012).

However, the importance of protein oxidation and subsequent changes in muscle food quality during storage time and processing has been shown earlier (Bertram et al., 2007; Lund, Hviid, & Skibsted, 2007; Ooizumi & Xiong, 2004). Several investigations have demonstrated that protein oxidation in relation to duration of frozen storage leads to loss of protein functionality (Badii & Howell, 2002; Herrero, Carmona, & Careche, 2004; Srinivasan & Hultin, 1994).

In addition, several authors have reported about the interaction between the primary and the secondary products of lipid oxidation with proteins during the storage time (Estévez & Cava, 2004). Some products of lipid oxidation, such as 4-hydroxynonenal and malondialdehyde (MDA), are capable of interacting with proteins, resulting in damage to the protein (Tironi., Tomas, & Anon, 2002). This interaction

may affect the functional properties of the proteins, increase liquid and nutritional compound loss during thawing, and decrease the firmness of the fish fillet.

The freezing of fish is one of the best ways of maintaining the inherent nutritional and sensory attributes and has been widely used by the industry for a long period of time (Ortiz, Palma, González, & Aubourg, 2008). Some authors have shown that storage of fish under ideal conditions can retain a taste, color, and firmness comparable to fresh fish for up to 3 months (Nielsen, 2007). Tokur et al. (2006) showed that in washed and unwashed minced mirror carp, significant changes in thiobarbituric acid-reactive substances (TBARS) were not reported after 5 months of storage at −18 °C. However, the acceptance of frozen stored fish by the consumer gradually declines, and texture is considered to be one of the major factors affecting the overall quality of fish (Subbaiah et al., 2015). Guo, Kong, Xia, Yu, and Liu (2014) investigated the effects of freeze–thaw cycles on common carp muscle and reported that the change in protein structure is responsible for reducing the quality of muscle. Through ice crystal formation and lipid–protein oxidation, liquid loss and lightness (L^*) can be increased, and the firmness, FA composition, sensory parameters, and color (redness [a^*]) can decrease in the fish fillet.

The main objectives of this study were to study the development of protein oxidation and lipid oxidation and also to monitor some alterations in nutritional quality, sensory score, liquid loss, and firmness of the common carp (*Cyprinus carpio*) fillet owing to the effects of frozen storage for up to 6 months.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Eighty individual market-size common carp (average weight, 2.1 kg) that had been reared under semi-intensive conditions in the same pond and purged for 1 month were obtained from the Trebon Fisheries Company in October, 2015. The fish were immediately transported to the processing facilities of the Institute of Aquaculture and Protection of Waters (IAPW), Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia (USB) in Ceske Budejovice, Czech Republic. The fish were stunned, slaughtered, gutted, washed, and filleted at the faculty premises by a trained person. At each time point, six fish were used, and both whole fillets from each fish (right and left fillet) were packed into plastic bags, labeled and frozen at −20 °C (the left fillet of each fish was used for the sensory evaluation test, and the right fillet was used for chemical analyses). Sensory and chemical analyses were executed at 0 time and after 1, 2, 3, 4, 8, 16, and 24 weeks. Regarding the chemical analysis, the six whole fillets of frozen fish from each time point were defrosted (kept at +4 °C overnight) and minced separately to obtain homogeneous and representative samples that were stored at −80 °C until further analysis. In the case of sensory evaluation, the whole fillets from each time point were defrosted (kept at +4 °C overnight) and used for sensory evaluation. In addition, 21 fillets were frozen at −80 °C as zero controls ($n = 3$) for the sensory evaluation at each sampling point.

2.2 | Water content and ash

Water content and ash content of fish fillets were determined in duplicate. Water content was determined by the weight loss after 4 hr at 60 °C in an assisted air circulation oven, followed by 8 hr at 105 °C. The ash content was determined at 550 °C (Method 923.03) according to AOAC (1995).

2.3 | Lipid content, lipid classes, and fatty acids

Lipid extraction was performed according to the methods by Hara and Radin (1978), with a slight modification. The samples were semi-thawed, and subsamples of approx. 1 g of fish fillet were taken for the extraction. The samples were homogenized for 3 × 30 s in 10 mL of hexane : isopropanol (3:2 v/v) using an Ultra Turrax (T25; IKA-Werke Janke & Kunkel GmbH & Co., Staufen, Germany), and 6.5 mL of Na_2SO_4 solution (0.47 M) was added. The homogenate was left to separate at 4 °C for 20 min and the upper phase was then transferred to a new tube and evaporated under N_2. The lipid content of the samples was determined gravimetrically from this total extracted lipid, which was then dissolved in 1 mL of hexane. Fatty acids from the total lipids were methylated with a boron trifluoride–methanol complex (BF_3) (Appelqvist, 1968). To each sample, 2 mL of a 0.01 M solution of NaOH in dry methanol was added, and the samples were then heated for 10 min at 60 °C. Then, 3 mL of BF_3 reagent was added, and the samples were reheated at 60 °C for 10 min. Then, the tubes were cooled in ice water and 2 mL of a 3.42 M NaCl solution in water was added to all tubes. The FA methyl esters (FAMEs) were extracted with 2 mL of hexane and the upper layer was transferred to a new tube and evaporated under nitrogen to dryness. The lipids were dissolved in 0.5 mL of hexane and stored at −80 °C until gas chromatographic (GC) analysis. The FAMEs were then analyzed using a gas chromatograph (Trace Ultra FID; Thermo Scientific, Milan, Italy) equipped with a flame ionization detector and a PVT injector, using a BPX 70 column (length, 50 m; *i.d.*, 0.22 mm; and film thickness, 0.25 μm; SGE Inc., Austin, Texas). The GC was programmed with a constant gas flow of 1.2 mL/min and a temperature program was started at 70 °C for 0.5 min, followed by a ramp of 30 °C/min up to 150 °C, a second ramp with a rate of 2 °C/min up to 220 °C, and a final constant time of 11 min at 220 °C. The injector and detector temperatures were set at 150 and 250 °C, respectively. The injector was set in the splitless mode, with a splitless time of 0.8 min and a split flow of 25 mL/min. The peaks were identified by comparing their retention times with those of the standard mixture GLC-68D (Nu-Chek Prep, Elysian, Minnesota) and other authentic standards (Nu-Chek Prep, Elysian, Minnesota; Larodan, Sweden).

For lipid classes, precoated silica gel 60 TLC plates (20 cm × 10 cm; 0.20 mm layer; Merck, Darmstadt, Germany) were used as the stationary phase. The analysis was performed according to the methods described by Olsen and Henderson (1989) with minor modifications. The samples were prepared at a concentration of 1 μg/μL and applied to the TLC plates with a Camag ATS 4 automatic TLC sampler. The lipid classes were separated by placing the TLC plate in a chamber with hexane–diethyl ether–acetic acid (85:15:1, v/v/v) as the mobile

phase for 15 min. After drying, the plate was sprayed heavily with copper acetate–phosphoric acid solution (3 g) $(CH_3COO)_2$ Cu, 8 mL of concentrated phosphoric acid, and 92 mL of ddH_2O. The plate was then developed at 160 °C for 15 min and cooled to room temperature. The proportions of the different classes of lipids were measured densitometrically using a Camag TLC scanner 3.

Lipid classes were identified by comparing the samples with an external standard (TLC 18-4A; Nu-Check Prep, Elysian, Minnesota). For data filtering, the Savatisky-Golay 7 mode and manual baseline correlation were used.

2.4 | Firmness analysis

Firmness analysis was performed instrumentally using a firmness analyzer (TA-XT. Plus, Stable Micro Systems, United Kingdom) by pressing a flat-ended cylinder (diameter, 75 mm; type P/75) into the section of a fillet below the dorsal fin perpendicular to the muscle fibers at a speed of 2 mm/s until the fillet was compressed to 50% of its original thickness. Firmness was defined as the maximum force detected during the first compression, expressed in grams.

2.5 | Fillet color

Fillet color was evaluated by the Minolta Colorimeter (Spectro Photo Meter, CM-600d, Konica, Japan) as described by Folkestad et al. (2008). The parameters L^*, a^*, and b^* were used to study the color properties of the fillet surface, where L^* is the luminance score and ranges from 0 (black) to 100 (white) and a^* and b^* are chromatic scores with negative to positive values representing green to red and blue to yellow, respectively. Each fillet was analyzed in five different regions.

2.6 | Sensory analysis

Sensory evaluation was performed by a sensory panel of 10 members of university employees from the University of South Bohemia, Faculty of Fisheries and Protection of Waters (Vodňany, Czech Republic) with experience in sensory evaluation of fish and fish products for raw and cooked carp. For this test, panelists were informed prior to the test to avoid eating spicy food, drinking coffee, being too full or too hungry, wearing strong perfumes, and smoking. Sensory evaluations were performed in individual cubicles to separate the panelists from each other (ISO 8589, 2007). Fillets were defrosted overnight in a refrigerator at 4 °C.

Panelists received cooked samples of the fillets stored at −20 and −80 °C. Fillets stored at −80 °C served as a reference sample for each time point. We assumed that at −80 °C, there were only minor chemical or enzymatic reactions; therefore, it was considered comparable to day 0 and used as a control to facilitate the comparison for the panelists during the long-time intervals.

Tasting samples consisted of three small pieces of fillet (2 × 2 cm). The samples were placed individually in 0.20 L glass jars and labeled lids with three random digit codes, then cooked for 15 min at 150 °C in an electric oven. For each group, samples were served warm (40–50 °C) in unopened jars. Panelists had to evaluate four parameters, namely, flavor, aftertaste, odor, and consistency. The scale of the various properties was from 0 to 100, where 0 indicated the Worst and 100 the Best evaluation for each attribute. The scale was subjective, meaning that the panelists gave their personal evaluation. If the product was not acceptable at all, the panel members were asked to state this, and the sample would be classified as unacceptable. However, this was not the case on any of the sampling days.

In addition, the panel members received a whole raw fillet which was stored at −20 °C and one that was stored at −80 °C as a control and a questionnaire, referring to color and firmness properties. The attributes to evaluate were firmness, odor, color, and overall acceptability. The scale used was from 1 to 5, where 1 indicated the worst and 5 the best quality.

2.7 | Thiobarbituric acid-reactive substances

The analysis of TBARS was conducted according to Miller (1998). The semifrozen samples were minced, connective tissues and visible fat were removed, and a subsample of approximately 1 g of muscle tissue was taken for analysis. The samples were homogenized with 9.1 mL (0.61 mol/L) of trichloroacetic acid (TCA) solution and 0.2 mL (0.09 mol/L) of butylated hydroxytoluene in methanol, using an UltraTurrax T25 (IKA-Labortechnik, Janke & Kunkel, Staufen, Germany) for 3 × 20 s at a speed of approximately 14 000 rpm. Then, the homogenate was filtered through a Munktell paper 00k (Munktell Filter AB, Grycksbo, Sweden). The filtrate (1.5 mL) was transferred twice to two new tubes; then, 1.5 mL of thiobarbituric acid (TBA) solution (0.02 mol/L) was added to the first tube (test sample), and 1.5 mL water was added to the second tube (sample blank). After reaction in darkness overnight at room temperature (20 °C), the reaction complex was analyzed at a wavelength of 530 nm against the sample blank using a UV-visual spectrophotometer (Specord 210; Analytik Jena, Germany).

2.8 | Peroxide values

Air was expelled from a conical flask using nitrogen gas. Fish oil (1 g), obtained as described above, was weighed into the flask, followed by 10 mL of chloroform and 15 mL of acetic acid. Potassium (1 mL) was subsequently added, the flask was sealed and swirled to mix the solution, and placed in a dark cupboard. Water (35 mL) was added after 15 min, and 1 mL of starch solution in water (1% w/v) was used as an indicator. The mixture was titrated with 0.0025 M of sodium thiosulfate solution. A control sample without the fish oil was also analyzed beside the sample. The peroxide value was calculated as follows

$$\frac{1,000\,(a-b)t}{w}$$

where a is the volume (mL) of sodium thiosulfate solution used in the sample, b is the volume (mL) of sodium thiosulfate solution used in the blank, t is the concentration (M) of sodium thiosulfate solution, and w is the amount (g) of sample used.

2.9 | Protein oxidation (2,4-dinitrophenylhydrazine)

Protein oxidation was estimated as carbonyls after incubation with 2,4-dinitrophenylhydrazine (DNPH) in 2 N of hydrochloric acid, following a

slightly modified method described by Levine et al. (1990). A fish sample (0.5–1 g) was homogenized in 10 mL of KCl (0.15 M) using an UltraTurrax T25 (IKA Labortechnik, Janke & Kunkel, Staufen, Germany) for 3 × 20 s at a speed of approximately 14 000 rpm. For spectroscopic determination of carbonyl groups, 100 μL of the homogenate was precipitated with 1 mL of TCA (10%). After centrifugation (5000 rpm, 5 min), the supernatant was removed. Then, the pellet was incubated with 1 mL of DNPH in HCl (2 M) and 1 mL of HCl (2 N) as a blank sample in the dark for 1 h with vortexing every 20 min. The samples were precipitated with 1 mL of TCA (10%), and the tubes were centrifuged in a tabletop microcentrifuge (5000 rpm) for 5 min, and the supernatant was discarded. The pellets were centrifuged with 1 mL of ethanol–ethyl acetate (1:1) at 10 000 rpm for 5 min. Then, the pellets were washed two times with 1 mL of ethanol/ethyl acetate (1:1, v/v). The pellet was redissolved in 1.5 mL of guanidine solution (6 M) and centrifuged in the microcentrifuge for 2 min at 5000 rpm. The spectrum was read against the complementary blank in the case of samples or against water in the case of purified proteins. The carbonyl concentration was analyzed as DNPH calculated on the basis of absorption of 21.0/(mM·cm) at 370 nm for protein hydrazine. Protein concentration was measured at 280 nm in the same sample and quantified by using bovine serum albumin as a standard.

2.10 | Liquid loss

Liquid loss was measured using a firmness analyzer (TA-XT Plus, Stable Micro Systems, United Kingdom) by pressing a flat-ended cylinder (diameter, 75 mm; type P/75) into the fillet below the dorsal fin perpendicular to the muscle fibers at a speed of 2 mm/s until the cylinder reached 50% of the fillet height and held for 60 s. A dry, preweighed filter paper (seven filter papers for each sample) was placed under the sample. The filter papers were then weighed immediately after the test, with and without the fish piece, and the water loss was calculated (Sampels, Asli, Vogt, & Morkore, 2010).

2.11 | The value of pH

Minced fish fillet (10 g) was homogenized with 20 mL of distilled water using a digital Ultra-Turrax T25 (IKA Labortechnik, Janke & Kunkel, Staufen, Germany). The homogenized sample was filtered using one filter paper, and the pH of the supernatant was measured using a digital pH meter (Testo 206) with a thermometer (Testo AG, Lenzkirch, Germany).

2.12 | Statistical analysis

Statistical evaluation of the chemical parameters and firmness was performed using one-factor ANOVA analysis in the Statistica CZ 12 software package. For sensory evaluation, nested ANOVA with sample nested either by date or by temperature and evaluator in the nested sample was used. In both cases, the level of significance was considered as $p < 0.05$, and the results are presented as the mean ± *SD*

In addition, a principal component analysis (PCA) plot was obtained using The Unscrambler v. 9.8 (Camo Process A/S, Oslo, Norway) for the oxidation parameters (MDA, PV, and carbonyls), liquid loss, pH, and the firmness and sensory data. Time points of week 3 and 2 months were excluded, as those data sets contained too many missing values and outliers. The values for firmness were weighted with 1/*SD*. Full crossvalidation was used as the validation model. The number of PCs finally used was determined to be 4.

3 | RESULTS

3.1 | Lipid content, water content, and ash content

The lipid content, water content, and ash content (g/100 g) of the samples at the different storage times are listed in Table 1. There were no statistical ($p < 0.05$) differences among the measured parameters.

3.2 | Lipid class and FA composition

The lipid class composition of the fish at the different storage times is summarized in Table 2. In comparison to the fresh samples, a significant ($F = 2.70$, $p < 0.05$) decrease in the combined monoacyl-(MAGs) and diacylglycerols (DAGs) during the storage time was found, but no major changes were observed in the relative percentages of phospholipids (PLs), cholesterol (CL), free fatty acids (FFAs), and triacylglycerols (TAGs) during the storage period.

The proportions of SFA, MUFA, and PUFA in the fresh fillets were 28.8, 59.3, and 12%, respectively (Table 3). In comparison to the fresh sample, there were no changes in FA composition over the storage period.

3.3 | Changes in the firmness, color, and sensory properties

A significant decrease in the firmness was found after 1 week of frozen storage ($F = 55.37$, $p < 0.05$) (Figure 1). Then, the values were constant. The lowest firmness was observed in the 4th week.

The color parameters for common carp fillets are shown in Figure 2. The initial values of L^*, a^*, and b^* were 47.51, −2.39, and

TABLE 1 Determination of ash, lipid, and water content (based on the fresh matter) of the common carp fillets stored for various times at −20 °C

Week	Fat content%	Water content%	Ash%
0	8.64 ± 2.36	71.8 ± 2.61	1.82 ± 0.17
1	12.9 ± 3.07	69.2 ± 2.64	1.58 ± 0.18
2	10.3 ± 2.02	72.0 ± 2.61	1.47 ± 0.26
3	9.48 ± 3.23	70.9 ± 3.42	1.43 ± 0.14
4	10.9 ± 3.76	70.0 ± 3.33	1.45 ± 0.48
8	11.8 ± 1.59	70.9 ± 2.31	1.25 ± 0.19
16	13.1 ± 4.21	69.1 ± 4.15	1.62 ± 0.2
24	10.8 ± 3.28	69.8 ± 2.83	1.66 ± 0.16

Averages ± *SD*, $N = 6$.

TABLE 2 Changes in the lipid class composition of common carp fillets during the storage time at −20 °C

Week	PL%	MAG + DAG %	CL%	FFA%	TAG%
0	12.92 ± 2.84	4.52 ± 0.84^{a}	5.67 ± 0.93	4.70 ± 1.60	72.16 ± 3.04
1	11.15 ± 1.9	3.15 ± 1.12^{ab}	4.43 ± 1.07	4.48 ± 1.47	76.76 ± 3.95
2	12.40 ± 3.6	2.93 ± 0.41^{ab}	4.91 ± 0.77	4.42 ± 1.22	75.30 ± 3.52
3	14.39 ± 2.25	3.38 ± 0.78^{ab}	4.86 ± 0.56	3.49 ± 0.74	73.85 ± 3.38
4	13.92 ± 3.29	2.93 ± 0.78^{ab}	4.14 ± 0.3	3.52 ± 1.25	75.47 ± 5.1
8	13.29 ± 1.49	2.57 ± 1.04^{b}	4.04 ± 0.41	3.19 ± 0.22	76.89 ± 2.4
16	12.69 ± 1.55	2.56 ± 0.45^{b}	4.33 ± 1.09	3.88 ± 1.76	76.52 ± 3.69
24	13.72 ± 1.93	2.5 ± 1^{b}	4.83 ± 1.24	3.66 ± 1.30	75.27 ± 4.63

Abbreviations (PL = phospholipids; MAG = monoacylglycerols; DAG = diacylglycerols; TAG = triacylglycerols; FFA = free fatty acids; CL = cholesterol)
Averages ± *SD*, *N* = 6, different superscript letters in a column are significantly different ($p < 0.05$) with respect to the period of storage.

4.10, respectively. After 24 weeks of frozen storage, the fillets tended to be lighter and more yellow than the fresh fillets. The L^* parameter increased significantly after the 3rd, 8th, and 16th week ($F = 6.31$, $p < 0.05$), but a^* showed only a slight increase after 8 and 24 weeks compared to day 0. The parameter b^* increased constantly and significantly after 3 weeks ($F = 8.61$, $p < 0.05$).

In comparison to raw fillets from day 0, raw samples that were stored at −20 °C showed a significant decrease ($F = 6.88$, 7.21, 7.03, and 2.86, $p < 0.05$) in all parameters until week 16, but there were no statistical differences in week 24 (Figure 3). Sensory evaluation of the whole raw fillet samples that were stored at −20 °C compared to −80 °C with respect to firmness, color, odor, and overall acceptability showed that in the 3rd week, the firmness and odor quality of the fish samples that were stored at −20 °C decreased significantly ($F = 1.70$, 1.85, 1.77, and 1.20; $p < 0.05$) compared to the fish samples that were stored at −80 °C. The comparison of the control sample (−80 °C) with samples from each time point in the 24th week showed a significant difference in all parameters ($p < 0.05$) (Figure 3).

TABLE 3 Changes in the fatty acid composition of common carp fillets during the storage time at −20 °C

Fatty acids	Fresh	Week 1	Week 2	Week 3	Week 4	Week 8	Week 16	Week 24
14:0	1.23 ± 0.10	1.22 ± 0.17	1.20 ± 0.10	1.10 ± 0.07	1.09 ± 0.09	1.09 ± 0.14	1.05 ± 0.11	1.06 ± 0.16
16:0	21.0 ± 1.58	20.1 ± 1.82	20.6 ± 1.58	20.2 ± 1.75	20.1 ± 1.41	20.0 ± 1.27	19.2 ± 1.75	19.2 ± 1.64
16:1	8.29 ± 0.78	8.29 ± 1.11	8.37 ± 0.54	8.27 ± 0.85	8.15 ± 0.72	7.95 ± 0.45	8.09 ± 1.11	8.41 ± 0.74
18:0	6.38 ± 0.77	6.59 ± 0.44	6.55 ± 0.68	6.92 ± 0.60	6.44 ± 0.44	6.95 ± 0.34	6.28 ± 0.63	6.23 ± 0.33
18:1*n* − 9	45.7 ± 4.04	48.3 ± 2.90	47.3 ± 3.25	47.0 ± 2.68	47.6 ± 3.26	49.1 ± 1.93	48.2 ± 4.09	49.4 ± 3.70
18:1*n* − 7	3.07 ± 0.20	3.16 ± 0.15	2.98 ± 0.18	3.19 ± 0.18	3.25 ± 0.16	3.04 ± 0.10	2.98 ± 0.21	3.16 ± 0.21
18:2*n* − 6	5.33 ± 0.66	4.34 ± 0.50	4.81 ± 0.51	4.53 ± 0.30	4.88 ± 0.93	4.51 ± 0.51	6.86 ± 6.26	4.56 ± 0.56
18:3*n* − 3	2.83 ± 0.94	2.13 ± 0.54	2.44 ± 0.57	2.36 ± 0.66	2.49 ± 0.67	1.95 ± 0.23	2.06 ± 0.64	2.20 ± 0.42
20:1*n* − 9	1.81 ± 0.23	1.87 ± 0.14	1.64 ± 0.09	1.84 ± 0.17	1.84 ± 0.13	1.82 ± 0.18	1.80 ± 0.21	1.94 ± 0.32
20:4*n* − 6	0.83 ± 0.23	0.65 ± 0.14	0.81 ± 0.26	0.85 ± 0.28	0.82 ± 0.26	0.64 ± 0.05	0.65 ± 0.13	0.77 ± 0.16
20:5*n* − 3	1.22 ± 0.40	1.08 ± 0.27	1.10 ± 0.28	1.25 ± 0.40	1.14 ± 0.31	0.96 ± 0.27	1.01 ± 0.29	1.08 ± 0.27
22:5*n* − 3	0.40 ± 0.10	0.37 ± 0.09	0.38 ± 0.10	0.47 ± 0.19	0.38 ± 0.08	0.33 ± 0.05	0.34 ± 0.10	0.37 ± 0.09
22:6*n* − 3	0.98 ± 0.32	0.77 ± 0.18	1.05 ± 0.35	1.15 ± 0.52	0.91 ± 0.26	0.72 ± 0.11	0.76 ± 0.23	0.91 ± 0.21
SFA	28.8 ± 1.26	28.0 ± 2.00	28.5 ± 2.08	28.4 ± 1.79	27.8 ± 1.76	28.2 ± 1.21	26.7 ± 2.11	26.6 ± 1.91
MUFA	59.3 ± 3.37	61.9 ± 2.72	60.7 ± 2.95	60.7 ± 3.19	61.26 ± 3.30	62.3 ± 1.70	61.4 ± 4.86	63.3 ± 3.43
PUFA	12.0 ± 2.49	9.59 ± 1.46	10.9 ± 1.75	10.9 ± 2.32	10.9 ± 2.47	9.53 ± 0.97	12.0 ± 6.75	10.2 ± 1.62
n − 3	5.59 ± 1.76	4.45 ± 0.97	5.09 ± 1.26	5.37 ± 1.76	5.05 ± 1.28	4.06 ± 0.54	4.29 ± 1.26	4.66 ± 0.95
n − 6	6.38 ± 0.88	5.14 ± 0.61	5.78 ± 0.76	5.55 ± 0.58	5.89 ± 1.22	5.46 ± 0.73	7.67 ± 6.29	5.49 ± 0.71
n − 6/*n* − 3	1.20 ± 0.23	1.19 ± 0.19	1.18 ± 0.27	1.09 ± 0.20	1.19 ± 0.14	1.36 ± 0.24	1.81 ± 1.26	1.20 ± 0.11

Averages ± *SD*, *N* = 6.

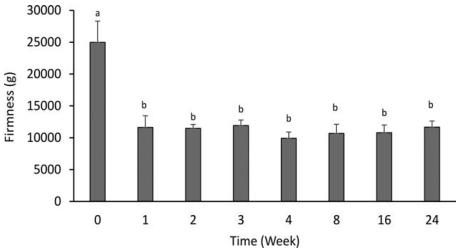

FIGURE 1 Changes in firmness of common carp fillet during storage at (−20 °C). Data on firmness are expressed as gram (g). Different superscript letters on the bars are significantly different ($p < .05$) with respect to the period of storage

Regarding the cooked samples, we could observe only small differences between the control sample (−80 °C) and the samples stored at −20 °C for the period of 6 months. In the 3rd week, flavor and aftertaste from the −20 °C samples decreased significantly compared to the samples stored at −80 °C ($F = 1.76, 1.38$; $p < 0.05$) (Figure 4).

Furthermore, with respect to odor, flavor, aftertaste, and consistency, comparing cooked samples that were stored at −20 °C with cooked samples from day 0 showed a significant decrease during the storage time, but the highest decrease was observed in week 3 ($F = 5.21, 8.37, 3.08$, and 2.99; $p < 0.05$) (Figure 4).

3.4 | Lipid and protein oxidation

The peroxide value increased significantly ($F = 9.34$, $p < 0.05$) from 1.57 meq O_2/kg oil on the first day to 2.12 meq O_2/kg oil in week 24 (Table 4).

Malondialdehyde increased significantly ($F = 8.15$; $p < 0.05$) in the 4th week (0.09 μg/100 g), then decreased to 0.04 μg/100 g in the 8th week, and stabilized at this level until the 24th week (Table 4). Protein oxidation, measured as carbonyl values, increased during the whole storage period (Table 4). After 2 weeks, the carbonyl content was significantly ($F = 5.03$, $p < 0.05$) higher than at the beginning of the experiment. However, after 3 weeks, the values were comparable to the first day. Then, values increased steadily.

3.5 | Liquid loss and pH

Liquid loss increased significantly ($F = 6.60$, $p < 0.05$) from the first day until 24 weeks of storage (Table 4). A significantly higher pH was observed in the 4th week, but pH values at the other times were similar to each other.

3.6 | Principal component analysis plot

The PCA correlation loadings plot was used to visualize the relationship between the chosen measured parameters (oxidation, sensory, firmness, pH, and liquid parameters) (Figure 5). PC 1 explained 91% of the variation, whereas PC 2 explained 5%. A clear correlation of the PV and the sensory parameters was demonstrated. Correlation loadings are mostly explained by PC 1 and are 0.748, 0.872, 0.984, 0.979, and 0.956 for PV, odor, flavor, aftertaste, and consistency, respectively. Liquid loss, however, was more correlated to the carbonyls, and both are explained mainly by PC 2, with correlation loadings of 0.623 and 0.548, respectively (Figure 5), showing that liquid loss is correlated to protein oxidation. Malondialdehyde was less correlated to the factors mentioned. The correlation loadings show that MDA is mainly explained by PC 3 (−0.567) where also pH has the highest explanation (−0.706) indicating some negative correlation between those factors. However, owing to the low explanatory percentage of PC 3 (3%), this explanation is not of significance.

4 | DISCUSSION

4.1 | Effects of storage on lipid, ash, and water content, FA profile, and lipid class composition

The lipid, ash, and water content of the fillets was comparable in this study showing no impact of freezing and storage period on these parameters. A small reduction in water content can be related to the evaporation, particularly at the low relative humidity that is maintained

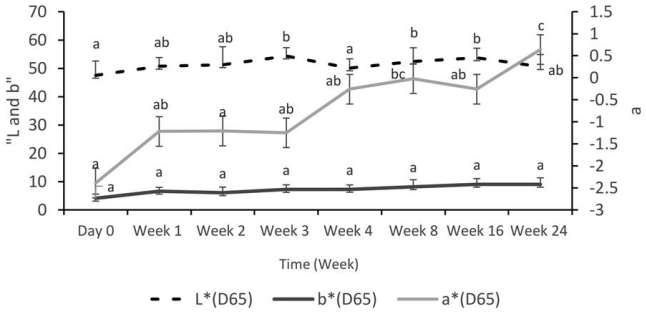

FIGURE 2 Colour changes during frozen storage at −20 °C of whole common carp fillet. Averages ± standard derivation, $N = 3$, different superscript letters above the lines are significantly different ($p < .05$) with respect to the period of storage

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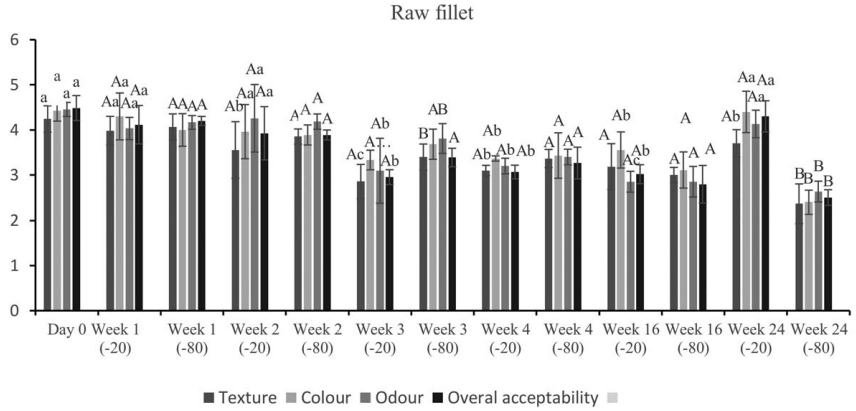

FIGURE 3 Sensory evaluation of the raw common carp fillets during frozen storage at −20 °C compared to the control samples (−80 °C) and day 0. Averages ± standard derivation, $N = 3$. Different capital superscript letters above a bar indicate significant differences ($p < .05$) between samples stored −20 and −80 °C at the same time point. Small superscript letters above a bar indicate significant difference ($p < .05$) between samples stored at −20 °C and day 0 for that parameter. 0 is the worst and 5 is the best value

in the freezer atmosphere. In addition, increasing the liquid loss owing to the effect of protein denaturation can be another factor (Goncalves, Rech, Rodrigues, & Pucci, 2008). Decreasing ash content might be linked to the drip loss during the defrosting process. Laila, Wahidu, and Tajul (2013) showed that the ash content decreased significantly during the chilled-frozen storage of bighead carp fillets. There is a correlation between proximate compositions such as fat and fillet firmness (Andersen, Thomassen, & Rora 1997). Investigation of those parameters during the storage time gives a better opportunity to find a correlation between firmness and fat content. In our study, we could not find any impact of fat content on firmness during the storage time.

In the lipid class composition, only a significant decrease in the combined MAG and DAG values was found, most probably the result of enzymatic degradation of these lipid classes. However, no increase in FFAs owing to the release of the FFAs from MAG and DAG was found, indicating an oxidation of the FFAs.

In contrast to our results, an increase of DAG in combination with a decrease of TAG and PL was found in hake (*Merluccius hubbsi Marin*)

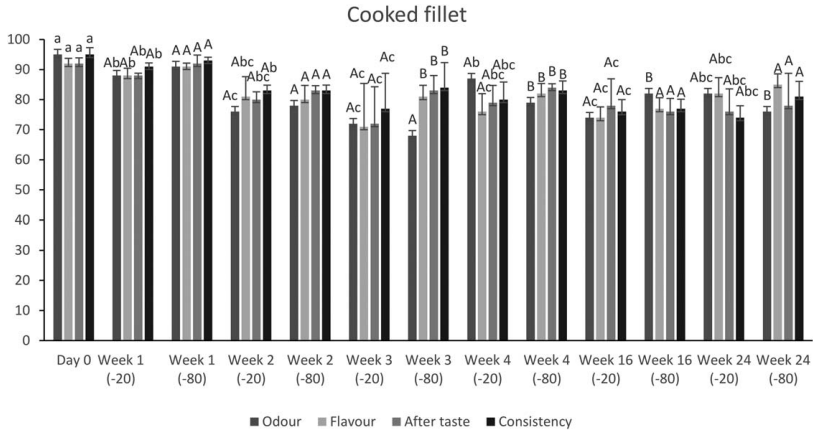

FIGURE 4 Sensory evaluation of the cooked common carp fillets after frozen storage at −20 °C compared to the control samples (−80 °C). Averages ± standard derivation, $N = 3$. Different capital superscript letters above a bar indicate significant differences ($p < .05$) between samples stored at −20 and −80 °C at the same time point. Small superscript letters above a bar indicate significant difference ($p < 0.05$) between samples stored at −20 °C and day 0 for that parameter

TABLE 4 Oxidation parameters in common carp fillets during frozen storage at −20 °C

	Week 0	Week 1	Week 2	Week 3	Week 4	Week 8	Week 16	Week 24
MDA (μg/g) ($n = 6$)	0.03 ± 00^{a}	0.04 ± 0.01^{ab}	0.05 ± 00^{ab}	0.06 ± 0.01^{b}	0.09 ± 0.03^{c}	0.04 ± 0.01^{ab}	0.05 ± 0.00^{ab}	0.04 ± 0.00^{ab}
PV(meq/kg) ($n = 3$)	1.57 ± 0.01^{a}	1.58 ± 0.01^{ab}	1.61 ± 0.005^{bc}	1.64 ± 0.01^{cd}	1.67 ± 0.01^{d}	1.91 ± 0.02^{e}	1.88 ± 0.01^{e}	2.12 ± 0.04^{f}
Carbonyls (nmol/mg)	2.33 ± 0.12^{a}	2.72 ± 0.06^{abc}	2.95 ± 0.15^{abc}	2.46 ± 0.20^{ab}	2.82 ± 0.16^{abc}	3.00 ± 0.52^{bc}	3.01 ± 0.55^{bc}	3.13 ± 0.44^{c}
pH ($n = 6$)	6.7 ± 0.12^{a}	6.44 ± 0.09^{a}	6.58 ± 0.16^{a}	6.65 ± 0.10^{a}	7.32 ± 0.44^{b}	6.65 ± 0.15^{a}	6.51 ± 0.13^{a}	6.35 ± 0.24^{a}
Liquid loss (%)	1.26 ± 0.52^{a}	3.93 ± 1.1^{ab}	3.92 ± 1.09^{ab}	3.78 ± 1.31^{b}	3.4 ± 0.72^{ab}	3.96 ± 1.21^{ab}	2.64 ± 0.67^{c}	5.58 ± 1.75^{b}

Abbreviations (MDA = malondialdehyde; PV = peroxide value)
Averages ± *SD*, $N = 3$, different superscript letters in a row are significantly different ($p < 0.05$) with respect to the period of storage.

and squid (*Illex argentinus*) during frozen storage for 120 days and 10 months, respectively (Paredi, Roldan, & Crupkin, 2006; Roldan, Roura, Montecchia, Borla, & Crupkin, 2005).

Regarding the FA composition, like our results, Polvi, Ackman, Lall, and Saunders (1991) did not find any modification in the total FA composition of salmon fillets during storage for 3 months at −12 °C, showing a low tendency for oxidative degradation.

4.2 | Changes in the firmness, color, and sensorial parameters

Also in line with our results, Vacha et al. (2013) investigated the textural factors (firmness, cohesiveness, and springiness) in the flesh of whole common carp that were stored at −20 °C for 84 days and found that all textural parameters mentioned had decreased. Especially, they found significant changes during the first month of storage. Additionally, Lu, Wang, and Luo (2017) reported a significant reduction in the texture of bighead carp (*Aristichthys nobilis*) with respect to hardness, springiness, gumminess, and chewiness during 28 days of storage at +4 and −3 °C. Gelman, Pasteur, and Rave (1990) showed a decrease in the texture of common carp (*C. carpio*) fillet during 40 days of storage at 0–2 °C. Cheng, Sun, and Zhu (2017) reported a decreasing trend in the hardness of grass carp (*Ctenopharyngodon idella*) fillets influenced by the freezing–thawing cycle.

In contradiction to our results, an increasing firmness in the first 3 months of storage, followed by a decrease, was reported in Atlantic herring (*Clupea harengus*) during storage at −25 °C (Szczepanik et al., 2010). At the same time, Subbaiah et al. (2015) reported a decrease in firmness during the first 4 months of storage at −18 °C and a subsequent increase for Nile tilapia (*Oreochromis niloticus*). Leygonie, Britz, and Hoffman (2012) also reported that the ice crystal formation during freezing time can cause damage to the ultrastructure in the fish muscle that may cause some biochemical reactions at the cellular level. In addition to the disruption of muscle microstructure by ice crystals, firmness is also influenced by enzymatic and oxidative protein degradation. As fish muscle contains high levels of indigenous proteases and causes protein degradation during processing and storage (Toyohara, Kinoshita, & Shimizu, 1990), and these contradicting results can be related to different protease activities in the different species. Myofibrillar proteins are the proteins most prone to denaturation, particularly myosin, as reported by Sharp and Offer (1992), in which during frozen storage, cod myosin reached up to 80% deterioration by comparison to the natural form of myosin. Carp fillets are less prone to degradation than marine fish owing to the absence of a trimethylamine system that

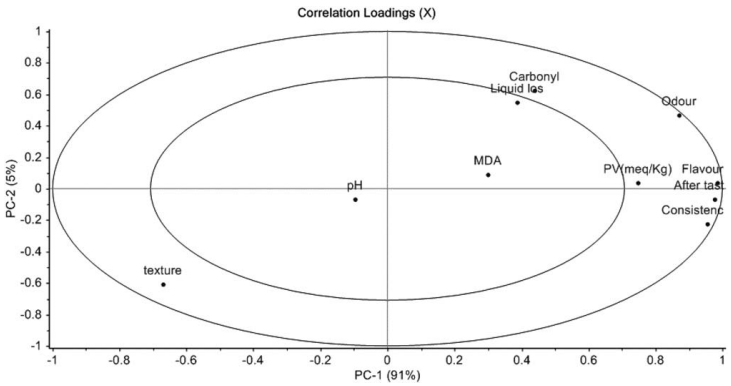

FIGURE 5 PCA plot showing the correlation loadings on PC 1 and PC 2 of the oxidation parameters MDA, PV, and carbonyls, pH, liquid loss, and the sensory and firmness parameters of common carp fillets at the time points 0, weeks 1, 2, 4, 16, and 24

is responsible for the formaldehyde formation, increasing protein denaturation and textural deterioration in the fish flesh during frozen storage (Vacha et al., 2013). The results of PCA also confirmed that in our study, protein oxidation had less impact on firmness. Therefore, we assumed that firmness was influenced more by frozen storage.

The increase in b^* and a^* values found in our study is in agreement with the findings by Yin, Luo, Fan, Wu, and Feng (2014), who described a significant increase in b^* and a^* parameters after storage of grass carp fillets at −40 °C for 12 hr and then at −20 °C for 5 days. Others (Suvanich, Marshall, & Jahncke, 2000; Wetterskog & Undeland, 2004) also reported a constantly increasing trend for L^* and a^* values during ice storage of washed cod muscle. Schubring (2005) reported a marked increase in L^* and b^* parameters in cod (*Gadus morhua*) fillets in the case of single frozen samples during 13 months storage at −20 °C and wave-like behavior for the a^* parameter. Additionally, Latip (2013) reported a significant decreasing trend for L^* and various changes for a^* and b^* parameters in bighead carp (*Hypophthalmichthys nobilis*) fillets during chilled-frozen storage. However, the high redness values in our study can be attributed to heme proteins that exist in the fillet of carp. The orientation of b^* to the yellow color during storage may also be related to the correlation of secondary products of lipid oxidation (aldehydes) in muscle with free amino groups that can generate some colored compounds called Schiff bases (Wetterskog & Undeland, 2004). An increasing L^* value shows the development of lipid oxidation during the storage time that can lead the color to lightness.

Regarding the sensory evaluation, for whole raw fillets that were stored at −80 °C and compared to −20 °C, the results, in general, also indicate a good stability concerning sensory aspects. The determined differences in the overall acceptability were not significant and quite small except for week 24. The slight increase of sensory parameters at the end of the storage time might be owing to some further reaction of volatile compounds that had built up. For the parameter firmness, the results actually correlate slightly with the instrumentally evaluated firmness data, which were, after an initial decrease until the 3rd week, stable during the whole period except for week 24. In addition, comparing −20 °C stored raw samples with day 0 samples showed a decreasing trend in all parameters during the storage time except in week 24. Decreasing sensory parameters indicated that the freshness in the samples had decreased. This trend could be due to the generation of lipid oxidation products.

However, comparing −20 and −80 °C cooked samples showed differences only in week 3 and after 4 weeks, indicating generally a good sensorial stability over the storage time.

However, the decrease of the mentioned parameters during the storage time at −20 °C in the cooked samples compared to the day 0 sample can illustrate a slight degradation during storage. The decrease of all parameters in the 3rd week may be related to some temporary difference in the panelists' reception.

4.3 | Correlation between lipid and protein oxidation products during the storage time

The peroxide values and TBARS values showed an increase during the storage time as a result of lipid oxidation as some authors have reported for Atlantic mackerel (*Scomber scombrus*) (Aubourg, Rodriguez, & Gallardo, 2005; Romotowska, Karlsdottir, Gudjonsdottir, Kristinsson, & Arason, 2016), horse mackerel (*Trachurus trachurus*), and Mediterranean hake (*M. mediterraneus*) (Simeonidou, Govaris, & Vareltzis, 1997).

The acceptable limit for peroxide values for human consumption in oils is 8 meq/kg (Boran, Karacam, & Boran, 2006), showing that our values never exceeded acceptable limits. Additionally, MDA values did not reach critical values >0.5 μg/g that are detectable by sensory properties (Lanari, Schaefer, & Scheller, 1995). Ke, Cervantes, and Roblesmartinez (1984) suggested that TBARS values <0.58 mg/kg were considered as nonrancid; 0.58–1.51 mg/kg as moderately rancid, but acceptable; and >1.51 mg/kg were considered as rancid. Both MDA and PV were in the acceptable range for human consumption. These results, together with the stable FA composition, confirm a good storage stability of the carp fillets at −20 °C up to 6 months.

In general, one would expect a steady increase in MDA over the storage time as it has been seen by several authors, for example in saithe (*Pollachius virens L.*) during 9 months of storage at −10 and −20 °C (Dulavik, SÝRensen, Barstad, Horvli, & Olsen, 1998), in *Wallago attu* fish during 30 days of storage at −12 °C (Gupta, Gandotra, Koul, Gupta, & Parihar, 2015) and in Spanish mackerel (*Scomberomorus commersoni*) during 6 months storage at −18 °C (Nazemroaya, Sahari, & Rezaei, 2009). Additionally, Jezek and Buchtova (2010) reported a significant increase in the amount of MDA and PV value during 11 days of storage in the normal package of common carp (*C. carpio L.*) fillets at +2 °C. In another study, Cheng et al. (2017) investigated the effect of freezing–thawing cycles on the grass carp (*Ct. idella*) fillets and showed a gradual increase in the amount of TBARS. The increased MDA values in the 4th week might be a result of peroxide decomposition. Although there were decreased MDA values in the 8th week, the decrease was most likely a result of a reaction between MDA and other compounds in the fish fillet. In line with our results, Aubourg et al. (2005) also found some decrease in the TBA index between the first and the 3rd month of storage, but thereafter, the values increased again until the 6th month. In our study, the MDA values remained stable. The formation of ice crystals during frozen storage in our study leads to cell disruption that can release pro-oxidants, such as heme-iron, and enhanced lipid oxidation also leads to the accumulation of the secondary products of lipid oxidation in the fish fillets. Furthermore, freezing at −20 °C can release the lipase enzyme from the lysosomes and hydrolyze the unsaturated FAs in the fish muscle.

Carbonyl values were significantly increased after 2 weeks. However, in the 3rd week, the carbonyl values decreased to 2.46 nmol/mg (this decrease might be related to the carbonylated protein degradation), but then rose constantly to the values of 3.13 nmol/mg after 6 months. The increasing carbonyl concentration shows a development of protein oxidation during the storage time. In a longer study of frozen storage of rainbow trout at −20 °C, after 2 years the amount of protein carbonyls increased to 5.2 nmol of carbonyl/mg (Baron, Kjaersgard, Jessen, & Jacobsen, 2007). During the first 6 months of storage, the carbonyl concentration increased >3 nmol of carbonyl/mg. Between the 8th and 13th month, a sharp increase in carbonyl concentration was observed. Lu, Wang, and Luo (2017) investigated the effects of

two different temperatures (+4 and −3 °C) on bighead carp (*A. nobilis*) fillets and reported that the carbonyl content of myofibrillar proteins increased at both temperatures but not significantly during 28 days of storage. Jasra, Jasra, and Talesara (2001) investigated the changes in myofibrillar protein of carp (*Labeo rohita*) with SDS-PAGE during 6 months of storage at −8 and −20 °C and revealed fading bands in the myosin light chain after 1 and 6 months of storage at −8 and −20 °C, respectively. Protein denaturation involves disruption of protein structures (secondary and tertiary) and could therefore result in changes in firmness. As unfolded proteins are more prone to ROS attacks, this could be the reason for the sharp increase in carbonyls after a certain initial storage time in the studies mentioned earlier as well as in our study (Baron et al., 2007). The formation of ice crystal during frozen storage appears to lead to partial dehydration of proteins, which causes protein denaturation in the fish fillets. In addition, interactions between oxidized lipids or formaldehyde and proteins are other reasons for protein denaturation.

The liquid loss increased by the effect of frozen storage indicates a decreasing water-holding capacity (WHC) of the fillets. Similar results on bighead carp, (*A. nobilis*) that was stored at +4 and −3 °C, showed that drip loss increased significantly after 12 days of storage (Lu et al., 2017). In agreement with our results, Yin et al. (2014) reported a significant increase in the amount of drip loss during 6 days storage of grass carp (*Ct. idellus*) by the effect of freeze-chilled process. Makri (2009) investigated the properties of WHC in gilthead seabream (*Sparus aurata*) fillets during 340 days of storage at −22 °C and reported the same trend. Additionally, similar results were published by Schubring (2005), who reported reduced WHC for cod samples stored at −10 and −20 °C up to 10 months. Bertram et al. (2007) reported that myofibrillar proteins lost their ability to retain water as a result of oxidation. Growth of ice crystals and increased ionic strength leads to protein aggregation. Denaturation/aggregation of actin and myosin might be responsible for the decreasing WHC (Subbaiah, 2015). The results of PCA confirmed that in our study, protein degradation influenced the increasing liquid loss.

Regarding pH, similar results to ours were reported (Eun, Boyle, & Hearnsberger, 1994). The pH can change the rate of lipid oxidation, but in this study, the pH value did not change during the storage time. Mozuraityte, Kristinova, Rustad, and Storro (2016) showed that when the pH was approximately 7, the values of PV and TBARS were lower compared to the lower pH, most likely because iron is needed to accelerate lipid oxidation, and at a higher pH, the amount of active iron is decreased, possibly another reason that the amounts of lipid and protein oxidation in this study were quite low during the whole storage time. Chen and Waimaleongoraek (1981) stated that there is a correlation between pH and lipid oxidation, which means that lower pH is linked to higher lipid oxidation. Wei et al. (2017) reported that the solute concentration is closely linked to the pH and lipid oxidation that can change the electrical properties and suggested that there is a relationship between the deterioration quality of meat and the electrical property changes.

Recently, several authors have reported interaction and a positive correlation between lipid and protein oxidation in fish, beef, and chicken (Estévez & Cava 2004). Most likely, lipid and protein oxidation start together and form oxidation products that can enhance either of them, resulting in an interaction of the oxidation processes. Torres-Arreola, Soto-Valdez, Peralta, Cardenas-Lopez, and Ezquerra-Brauer (2007) demonstrated that lipid oxidation leads to some alterations in fish fillet firmness due to lipid–protein interaction during frozen storage. Lipid–protein interaction can change the interactions between protein and water that can affect the integrity of the fillet fibers. In this case, some carbonyls from the oxidized lipids can participate in covalent bonding with proteins, and this reaction leads to the formation of permanent protein–lipid aggregates (Zirlin & Karel, 1969). In the carbonylamine reaction, some products of lipid oxidation such as aldehydes can bind directly to amino groups in the protein or through covalent bonds (Gardner, 1979). According to our results, after 6 months of storage of common carp fillets at −20 °C, the development of oxidation of lipids and protein was not very intense, and we could not observe correlation between them. However, the results showed that the lipid and protein oxidation had a similar tendency.

4.4 | Principal component analysis

The results of PCA indicated that the WHC is more correlated to protein degradation than to lipid oxidation. Firmness, on the other hand, is negatively correlated to all other parameters (correlation loading −0.670), confirming our conclusion that the freezing itself has the highest impact on this factor but not the ongoing degradation during the storage time. As already mentioned, pH is mainly explained by PC 3 and is situated close to the center of PC 1 and PC 2 with very low negative correlation loadings, indicating that it does not have a significant impact on oxidation, firmness, and sensory traits. Regarding the relationship of lipid oxidation to sensory parameters, the low correlation in PC 1 and PC 2 is in line with the conclusion that the values were below detectable levels for sensory evaluation.

5 | CONCLUSIONS

This study illustrated that the development of lipid and protein oxidation in fish stored at −20 °C seems to have a similar tendency, but lipid oxidation was faster in the beginning of the storage period compared to protein oxidation. Together with lipid and protein oxidation, liquid loss increased over time, also indicating an increasing protein denaturation. However, neither PV nor MDA values reached critical levels, indicating a minimal oxidation during storage for 6 months at −20 °C. This conclusion was underlined by no changes in the FA composition and only minor changes in lipid class composition. Firmness was decreased owing to freezing but not owing to storage time. We could show a correlation between protein oxidation and liquid loss, whereas lipid oxidation was more related to sensory aspects.

We observed a minimal lipid and protein oxidation in the normal packaging and temperature that are used in the domestic freezers. In general, a high nutritional value and a good storage stability of carp fillets for at least up to 6 months were demonstrated.

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CHAPTER 4

PROTEOME CHANGES OF EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FILLETS FROM TWO DIFFERENT REARING SYSTEMS DURING FROZEN STORAGE

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PROTEOME CHANGES OF EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FILLETS FROM TWO DIFFERENT REARING SYSTEMS DURING FROZEN STORAGE

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ABSTRACT

Proteome analysis as well as chosen quality parameters were used to investigate the quality of fillets from Eurasian perch reared in either recirculating aquaculture system (RAS) or pond during 10 months storage at -20°C. The band intensity in a SDS-PAGE analysis decreased while western blot indicated an increasing trend in the level of protein carbonyls in both groups. Concerning SDS-PAGE result pond fillet (PF) showed relatively higher loss over time of storage compared to the RAS fillet (RF) that showed lower initial intensity bands. Native page structure showed higher molecular weight proteins in RF compared to PF. Quantitative analysis of protein carbonyls showed the same pattern as the western blotting while no significant difference was observed in malondialdehyde (MDA) values between PF and RF. Firmness and calpain activity decreased while liquid loss increased significantly during storage however, RF showed higher firmness and liquid loss compared to PF after 10 months.

Keywords: *proteome analysis, fish, protein oxidation, firmness, liquid loss*

Abbreviations: *Recirculating aquaculture system (RAS), fillet from RAS (RF), fillet from pond (PF)*

Introduction

The quality of fish flesh can be influenced by several parameters, such as physical condition, genomics, environment, pre-/in-/post- rigor mortis conditions, for example, time and storage temperature. Fillet of Eurasian perch (*Perca fluviatilis* L.) is a highly regarded product and has a high price in the market (Watson, 2008). The main production of perch is traditional pond polyculture or wild catches.

Through the last two decades recirculating aquaculture systems (RAS) has been expanded for the difference of European inland aquaculture (Tryggvason, 2016) . In this rearing system stable temperature (23°C), uniform photoperiod (12L:12D), high stocking density (up to 60 kg m-3) and also commercially formulated feeds are used (Fiogbé & Kestemont, 2003). Stocking density can be considered as an important factor to evaluate the efficiency and profitability of commercial fish farms (Mairesse, Thomas, Gardeur, & Brun-Bellut, 2007). However, rearing system might have direct or indirect impact on some fish flesh factors, such as fibre development, colour and textural parameters.

The deterioration of fish fillet is much faster compared to other muscle foods. Therefore, the storage conditions are important. Freezing is used as a common method in order to keep the nutritional and sensory quality of fish flesh for a long storage time (Ortiz, Palma, González, & Aubourg, 2008). Nielsen (2007) reported that under ideal conditions fish can be kept for up to three months without any comparable changes to fresh fish in the colour, taste and firmness. In the previous study, we demonstrated a good storage stability at -20°C for common carp for up to 6 months (Hematyar, Masilko, Mraz, & Sampels, 2018). However, fluctuating temperature during long term storage at -10 and -20°C lead to a damage in the fish fillet (Kjaersgard, Norrelykke, & Jessen, 2006). Rancidity can be increased in fish fillet due to high amount of polyunsaturated fatty acids (PUFA), which are prone to oxidise (Kjaersgard, et al., 2006). On the other hand, protein denaturation and calpain activity, which is responsible to decrease water holding capacity (WHC) and tenderness, also highly impact the fish quality (Mackie, 1983). It is assumed that actin and myosin are the main responsible proteins for the functional properties of muscle foods and during frozen storage both proteins can undergo aggregation reaction and decline WHC and firmness in the muscle (Mackie, 1993). Hence, the development of lipid and protein oxidation may be considered as important parameters and both are responsible for colour, texture and nutritional value and thus the muscle foods quality.

The objective of this study was to determine the fillet quality of Eurasian perch, which was reared in two different rearing systems influenced by long term frozen storage by the proteomic approach and also by respect to lipid oxidation, firmness, WHC and calpain activity parameters. Furthermore, the interrelated lipid-protein damage was in focus.

Materials and Methods

Experimental design

Forty eight market size Eurasian perch (average 183 g and 211 g for 30 fish in weight for RAS and pond fish respectively) with the pond age 2+ and RAS age 1+, from two different rearing systems (traditional pond culture and RAS) were used in the present study.

Pond reared perch were obtained from the fish farm Rybarstvi Nove Hrady Inc. (pond Zar) located in the south of the Czech Republic (48°8′N, 14°7′E). Fish were reared in polyculture (natural production of 240 kg ha^{-1}) with common carp (*Cyprinus carpio*) as a main fish species. The food source was natural prey with the main forage fish being topmouth gudgeon (*Pseudorasbora parva*), roach (*Rutilus rutilus*) and benthic organisms. The fish were harvested on 19 October during the common Czech harvest period.

Fish from RAS were reared at the faculty premises and fed commercial feed (Inicio plus, BioMar, France) with the nutrient values: protein 52%, fat 23%, fibre 0.9%, carbohydrates (NFE) 12%, gross energy 23.5 MJ kg^{-1}, digestible energy 20.6 MJ kg^{-1} (manufacturer's data).

Fish were kept at the faculty in tanks at the same temperature (20 °C) for one week before being slaughtered, gutted, skinned, washed and filleted on the faculty premises by one trained person. Fish fillet were randomly packed in plastic bags, labelled and stored in a freezer at -20 °C. Textural, chemical and proteomics analyses were executed on the first day and after 4, 8 and 10 months of storage.

Extraction of Muscle Proteins

Pieces of 100 mg of frozen fish muscle tissues were cut and weighed at -20°C to minimize artifactual protein degradation. The frozen muscle tissue was homogenized in 500 µl in 50 mM PBS, pH=7.4). Crude extracts were transferred to an Eppendorf tube.

SDS-PAGE

SDS-PAGE was performed according to the method of (Laemmli & Eiserling, 1968). Sample (20 µl) was mixed with Laemmli sample buffer with a final protein concentration of 2µg/µl, followed by heating for 2 min at 95 °C. Afterwards, the samples were loaded on to a 10% Criterion Tris glycine Gel (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis at a constant electrical potential of 200 V. As marker proteins, the Spectra Multicolor Broad range protein Ladder, (15–220 kDa) (Thermo Scientific, Rockford, USA) was used. Subsequently the electrophoresis the gel was stained with 0.5% Coomassie Brilliant Blue G-250 (Bull Korean Chem Soc. 2002).

Reaction with 2,4-DiNitroPhenyl Hydrazine and Immunoblotting

For immunoblotting the 2,4-dinitrophenyl hydrazine (DNPH) reaction was performed directly on the protein homogenate or on the sarcoplasmic protein fraction (low salt soluble protein) obtained by centrifugation of the protein homogenate at 12600 g for 3 min. The supernatant was used for analyse.

Protein carbonyls were derivatized by mixing 20 µL sample (1:1) with 12% SDS, 10% TFA, 10 mM DNPH and incubated for 30min at RT. The reaction was stopped by adding 40 µL neutralization buffer (1:1) containing 2M Tris-base, 30% Glycerol, 20mM DTE before separated on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Prior to DNPH reaction in immunoblotting the protein concentration was adjusted to 10 mg/mL by using the BCA kit (Pierce, Rockford, IL). The samples were centrifuged for 3 min at 12600 g and loaded on the gel (10% Tris glycine gels; Bio-Rad, Hercules, CA, USA). The protein bands were transferred to a polyvinylidene difluoride (PVDF) membrane 0.2 µm (Bio-Rad, Laboratories, USA) using Trans-Blot SD, semi-dry transfer cell, 0.35 A, max 50 V for 60 min (Bio-Rad, Laboratories, USA). After transfer, the membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) buffer (0.137 M NaCl and 20 mM Tris-HCl, pH 8.0) and incubated with a 1:16000 dilution of rabbit anti-DNP (Sigma Aldrich, Germany), in TBS overnight at +4°C. The membranes were washed in TBS and incubated in a 1:8000 dilution of the secondary antibody, peroxidase conjugated swine anti rabbit (DAKO Denmark A/S). After washing in TBS, the blot was either developed using the ECL± kit (Bio-Rad Laboratories, USA). Image analysis of gels and blots were performed using the software Image lab (Molecular Imager Chemi Doc XRS+, Bio-Rad Laboratories, USA).

Native PAGE

Sample was mixed with Lysis buffer with a final protein concentration of 5µg/µl. Then, incubated for 15 min on ice followed by centrifuge at 20000 × g for 30 min in room temperature. Samples (15 µl) for native PAGE analysis were used without heating treatment. Kit (Life Technologies) and the protein were separated on 3-12% Native PAGE gel (Life Technologies) using Native PAGE Running buffer kit (Life Techmologies). Gels were stained with colloidal CBB.

Protein identification by MALDI-TOF analysis

Bands of interest were excised from the gels are subjected to in-gel trypsin digestion. Briefly, excised spots were washed, reduced, S-alkylated and digested with trypsin (Promega, Nacka, WI, Sweden) as described by (Jensen, Larsen, & Roepstorff, 1998). Prior of analysis, the samples were desalted and concentrated using custom made C18 columns and eluted directly on the target plate using 10 g/l α-cyano-4-hydroxycinnamic acid (Sigma Aldrich, St. Louise, MO, USA), 1% formic acid and 70% acetonitrile.

The peptides mixtures were analysed by using MALDI -TOF MS/MS (Autoflex Speed, Bruker Daltonics, Bremen, Germany). For internal calibration a peptide standard mixture from 1000–3000 Da (Bruker Daltonics, Bremen, Germany). Protein identification was performed by using Peptide Mass Fingerprinting using the in house Mascot server according to (Wedholm et al., 2008).

Protein oxidation with 2,4-dinitrophenylhydrazine

Protein oxidation was estimated as carbonyls content after incubation with 2,4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid following a slightly modified method described by (Levine et al., 1990). 0.5–1 gram of meat will was homogenized in 10 mL KCl (0.15 M) using an UltraTurrax (Janke & Kunkel, Staufen, Germany, T25IKA-Labortechnik,) for 3 × 20s at a speed of approximately 14000 rpm. 100 µl of homogenate solution was pipetted into 2 mL centrifuge tubes (Eppendorf) and 1 mL trichloroacetic acid 10% (TCA) was added to each tube then, centrifuged at 5000rpm for 5 min. The supernatant was removed. 1 mL HCl (2N) to the blank and 1mL 2,4-dinitrophenylhydrazine in 2 M HC1 were added and allowed to stand at room temperature for 1 hour, with vortexing every 20 min. Then 1 mL trichloroacetic acid (10%) was added and centrifuged the tubes in a table top micro centrifuge (5000 rpm) for 5 min, and the supernatant was discarded. The pellets were centrifuged with 1 mL ethanol-ethyl acetate (1:1) in 10000 rpm for 5 min at 4°C. The pellets were washed 2 times with 1 mL ethanol-ethyl acetate (1:1) to remove free reagent. The precipitated protein was redissolved in 1.5 mL guanidine solution (6M) and centrifuged in the micro centrifuge for 2 min at 5000 rpm. The spectrum, read against the complementary blank in the case of cruder samples or against water in the case of purified proteins. The carbonyl concentration was analysed as DNPH calculated on the basis of absorption of 21.0 mM^{-1} cm^{-1} at 370 nm for protein hydrazine. Protein concentration was measured at 280 nm in the same sample and quantified by using bovine serum albumin as a standard.

Thiobarbituric acid reactive substances (TBARS)

Analysis of TBARS was conducted according to (Miller, 1998). The semi-frozen samples were minced, connective tissues and visible fat were removed, and a sub-sample of approximately 1 g of muscle tissue was taken for extraction. The samples were homogenized with 9.1 mL (0.61 mol/L) of trichloroacetic acid (TCA) solution and 0.2 mL (0.09 mol/L) of butylatedhy droxytoluene (BHT) in methanol, using an UltraTurrax (Janke & Kunkel, Staufen, Germany, T25IKA-Labortechnik,) for 3 × 20 s at a speed of approximately 14000 rpm. Afterwards the

homogenate was filtered through a Munktell paper (Munktell Filter AB, Grycksbo, Sweden). Two times 1.5 mL of the filtrate were transferred to new tubes and 1.5 mL of thiobarbituric acid (TBA) solution (0.02 mol/L) was added to the first (test sample) and 1.5 mL water to the second (sample blank). After reaction in darkness for 15–20 h (overnight) at room temperature (20°C), the reaction complex was detected at a wavelength of 530 nm against the sample blank using a UV-visual spectrophotometer (Specord 210; Analytik Jena, Germany). The amount of TBARS was expressed as malondialdehyde (MDA) (μg/g).

Firmness analysis

Firmness analysis was performed instrumentally using a firmness analyser (TA-XT. Plus, Stable Micro systems UK) by pressing a flat-ended cylinder (10 mm diameter, type P/10) into the section of fillet below the dorsal fin perpendicular to the muscle fibres at a speed of 2 mm s^{-1} until the fillet was compressed to 50% of its original thickness. Firmness was defined as the maximum force detected during first compression, expressed in grams.

Liquid loss

It was measured with a texture analyser (TA-XT. Plus, Stable Micro systems UK) by pressing a flat-ended cylinder (10 mm diameter, type P/10) into the fillet below the dorsal fin perpendicular to the muscle fibres at a speed 2 mm s^{-1} until it reached 50% of the fillet height and held for 60 seconds. A dry, pre-weighed filter paper was placed under the sample. The filter paper was then weighed immediately after the test, with and without the fish piece and the liquid loss was calculated.

Calpain activity assay

Calpain activity was measured according (Salem et al., 2005). Briefly, 100 μg of each fish fillet sample form both rearing systems were homogenized in extraction buffer and then the concentrate was diluted to a final protein concentration of 200 μg in 85 μl. 10 microliters of reaction buffer (10x) and 5 μL of calpain substrate were added follow by incubation at 37 °C for 1 h. Samples were measured using 400 nm excitation filter and 505 nm emission filter. The calpain activity change was expressed as relative fluorescence fold change.

Fillet colour

Fillet colour was evaluated by the Minolta Colorimeter (Spectro Photo Meter, CM- 600d, Konica, Japan) as described by (Folkestad et al., 2008). The parameters L* (lightness), a* (red-green spectrum), and b* (yellow-blue spectrum) were used to study the colour properties of the fillet surface, where L* is the luminance score and ranges from zero (black) to 100 (white) and a* and b* are chromatic scores.

Statistical analysis

Statistical evaluation was done by using one-factor ANOVA analysis in the Statistica CZ 12 software package. The level of significance was considered at $p<0.05$ and the results are presented as mean ± S.D.).

Results

Protein alteration during storage at -20 °C

In this study protein patterns of the fillets from RAS and pond systems during 10 month frozen storage at -20 °C were identified with SDS-PAGE analysis. The pattern showed several bands from 15 to 220 kDa in the fillets from both systems. The bands, which appeared at 200 kDa are myosin heavy chain (MHC), nebulin (107 kDa), actin (43 kDa), troponin (30 kDa) and myosin light chain components (25-15 kDa) (Seki & Watanabe, 1982). Beta-enolase (49 kDa), actin (43 kDa), GAPDH (37 kDa), LDH A-chain (37 kDa), and trisephosphate isomerase (28 kDa) were confirmed by MS.

The SDS-PAGE protein profiles indicated loss of protein during frozen storage. Loss of protein was observed during storage for both systems. At t=0-4 moth, the PF showed more intense bands than RF same time points. For both system the most proteins band disappeared or fainted during 10 months storage at -20 °C. MHC, α-actin, actin and MLC showed the highest degree of fainting during storage for both systems (Figure 1 A). Bands of serum albumin, troponin bands fainted less and only after 8 and 10 months of storage.

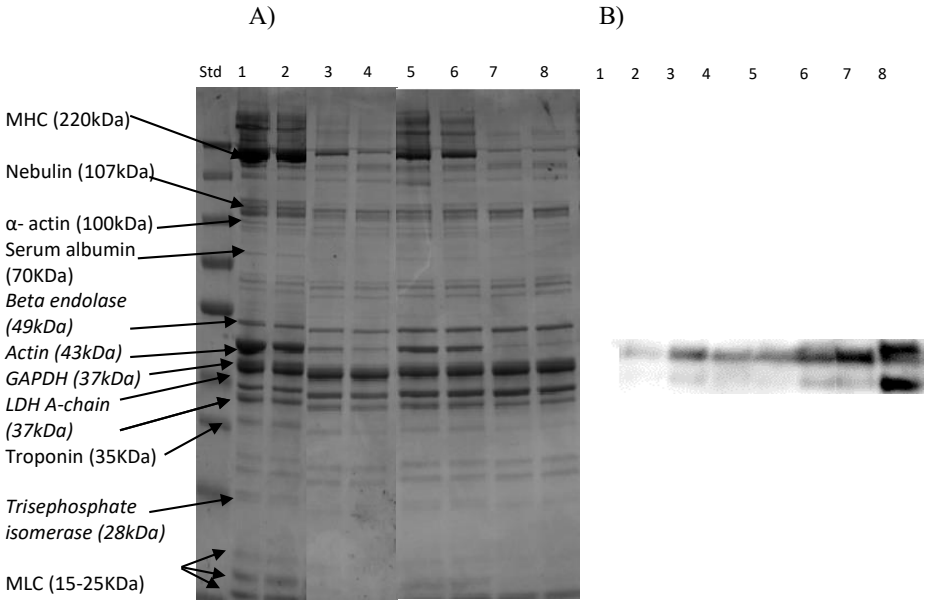

Figure 1. *(A) SDS-polyacrylamide gel electrophoresis and (B) immunoblotting against protein carbonyl groups in perch fillet from pond (line 1-4) and RAS (line 5-8) systems during frozen storage at -20°C. Fillets from pond fish: Line 1) t = 0, 2) 4 months, 3) 8 months and 4) 10 months; Fillets from RAS fish5) at t = 0, 6) 4 months, 7) 8 months, and 8) 10 months. Identified protein in regular text, protein given in italic were confirmed by MS.*

Western blot (immunoblot) of protein carbonyl groups in the fillets protein from both rearing systems showed that highly intense bands at 40, 43 corresponding to actin and a none-identified intensive protein and less intense bands at 37 kDa, which increased during storage (Figure 1 B). PF showed less oxidized carbonyls compared to the RF at t= 0 and 4 month and after 10 months RF showed higher content of oxidised carbonyls compared to the PF at the same time point. Results confirmed the development of protein oxidation in both fillets during the storage time.

MALDI-TOF analysis

The 5 protein spots of interest were identified by protein fingerprints (PMF) and using MALDI-TOF/TOF mass spectrometry (Table 1). According to the protein function from Peptide Mass Fingerprinting online Mascot server, the identified proteins were actin, GAPDH, LDH A-chain, Beta-enolase, Triose phosphate isomerase and Beta-enolase.

Table 1. *Protein identification by PMF (Peptide mass fingerprinting) and MALDI TOF/TOF.*

No.	ID	Acc.no.	Function	Mascot Score	Expectation	Peptides in match	Mass (kDa)	pI
#1	Actin	P68140	Skeletal muscle protein	127*	$1.1e^{-009}$	11	43	5.22
#2	GAPDH	Q5XJ10	Carbohydrate degradation; glycolysis	68*	0.00095	8	37	8.20
#3	LDH A-chain	O93543	Pyruvate fermentation to lactate	58*	0.009	7	37	6.35
#4	Beta-enolase	B5DGQ7	Carbohydrate degradation; glycolysis; pyruvate from D- glyceraldehyde 3-phosphate	63*	0.0027	8	49	6.61
#5	Triosephosphate isomerase	Q90XG0	Glycolysis Gluconeogenesis	79*	7.2e-005	8	28	6.45

Native-PAGE

Concomitantly, some high molecular polymers (protein aggregates, more than 200 kDa weigh), that stacked on the 1D gel, were observed by Native-PAGE. Analysis of the muscle proteins of fillets from both systems by native gel electrophoresis showed highly intensive bands around 1236 kDa at t = 0 month. The band of this high molecular weight protein was very week after 8-10 month of storage in the PF, whereas the highest loss of in RF was observed after 10 month. Thus, still quite intense after 8 month of storage in the RF. PF also showed less intense band 720 kDa after 8-10 month whereas no loss was observed in the RF after storage at -20 °C. The weak band around 480 kDa decreased in the PF at 8-10 months of storage whereas not changes were observed for the RF. The band at 25 kDa were faded after 8–10 months of storage for the PF, whereas only small changes were observed for the RF over time of storage with a small decrease in band intensity after 10 month of storage (Figure 2). Comparing the results between the RF and PF revealed more stability in the RF proteins during the storage time.

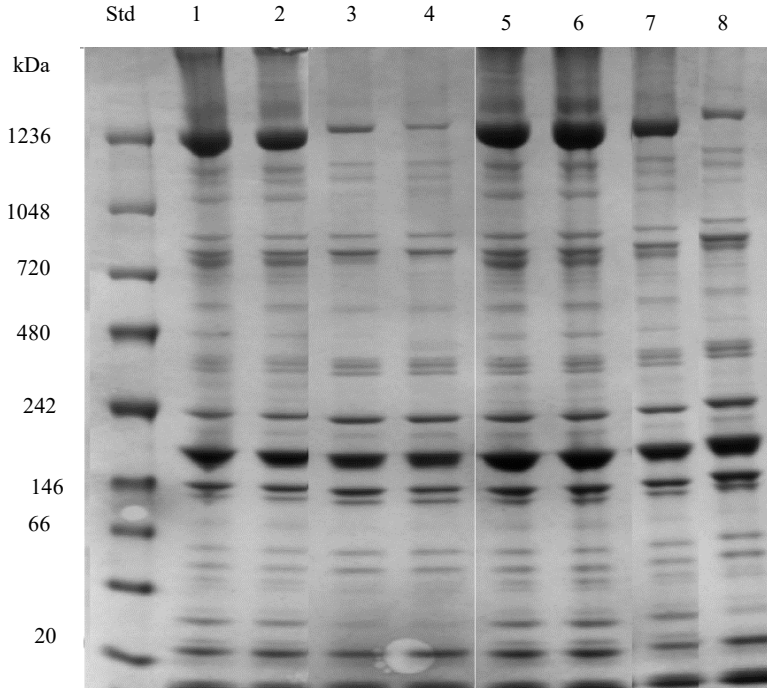

***Figure 2**. Native PAGE, Bis-Tris gel of perch fillet from pond (line 1-4) and RAS (line 5-8) systems during frozen storage at -20 °C. Line 1) t = 0, pond fillet, 2) 4 months, pond fillet, 3) 8 months, pond fillet, and 4) 10 months, 5) at t = 0, RAS fillet, 6) 4 months, RAS fillet, 7) 8 months, RAS fillet, and 8) 10 months, RAS fillet.*

The protein carbonyl contents enhanced significantly in the period of storage time in RF and the PF ($p < 0.05$) (Table 2). The amount of carbonyls in the first day was 1.90 and 0.47 (nmol/mg) that increased up to 4.38 and 3.94 (nmol/mg) in the RF and PF, respectively, after 10 months at -20°C. The amount of carbonyl in fillets from both rearing systems showed statistical differences between t=0 and t=4 and also in between the two rearing systems while after 8 and 10 months we did not observe any differences between the systems.

***Table 2.** MDA (µg/g) and Carbonyl content (nmol/mg) parameters in perch fillets from RAS and pond during refrigerated storage at -20°C (mean ± S.D., n=6).*

	MDA (µg/g)		**Carbonyls (nmol/mg)**	
Time (Month)	RAS	Pond	RAS	Pond
0	0.28 ±0.01^{Aa}	0.28 ±0.0^{Aa}	1.9 ±0.01^{Aa}	0.47 ±0.02^{Ab}
4	0.51 ±0.01^{Ba}	0.48 ±0.02^{Ba}	3.1 ±0.01^{Ba}	1.78 ±0.02^{Bb}
8	0.56 ±0.05^{Ba}	0.53 ±0.02^{Ba}	3.82 ±0.05^{Ba}	3.1 ±0.02^{Ca}
10	0.73 ±0.04^{Ca}	0.69 ±0.02^{Ca}	4.38 ±0.04^{Ca}	3.94 ±0.02^{Ca}

Different capital superscript letters in a column indicate significant differences ($p<0.05$) within each rearing system. Small superscript letters in a row indicate significant difference ($p<0.05$) between rearing systems at the same time point

Abbreviation: malondialdehyde (MDA)

Lipid oxidation

The amount of MDA in the fillets from both rearing systems is also presented in (Table 2). MDA increased significantly in RF and PF ($p < 0.05$) during the 10 months of frozen storage (-20°C). The initial amount of MDA was 0.28 MDA (µg/g) on the slaughtering day and increased to 0.73 and 0.69 MDA (µg/g) after 10 months in RF and the PF respectively. While, comparing MDA values in each time point RF and PF did not show any statistical differences during 10 months storage.

Furthermore, we observed a high correlation between TBARS and protein carbonyl content ($r=0.98$ and $r=0.97$) in the RF and PF respectively.

Firmness changes

The results of firmness was measured in perch fillets from both rearing systems (RF and PF) are shown in (figure 3). In both RF and PF firmness decreased significantly ($p < 0.05$) after 10 months storage at -20 °C. The initial firmness value were 1533 and 1239 and decreased to 790 and 518 in the RF and PF, respectively. Furthermore, the comparison from time to time between RF and PF firmness illustrated a significant ($p<0.05$) decrease in the firmness after 4 and 10 months storage. Generally, RF showed a higher firmness compared to PF in the period of storage time. Additionally, we observed a major reduction in firmness after 4 months storage in both PF and RF (43 and 40%) respectively however, in the rest of times we could not see this percentage of decline.

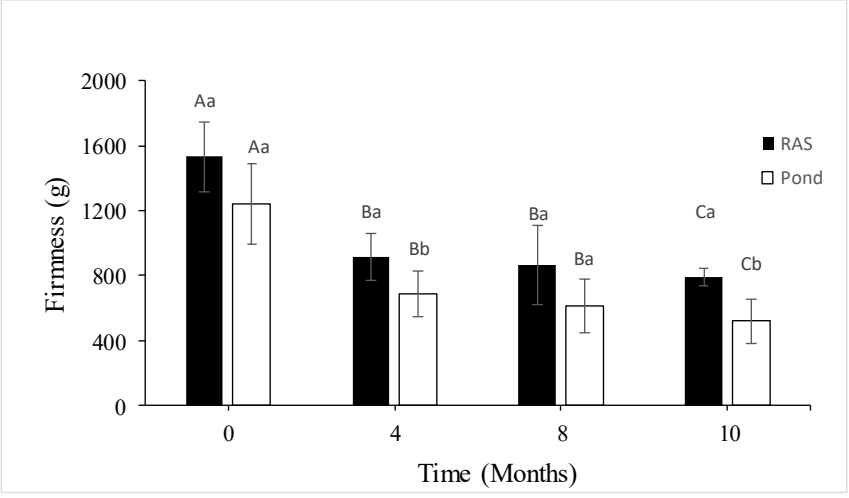

Figure 3. *Changes in firmness of perch fillet from RAS and pond system during storage at (–20 °C). Different capital superscript letters over a bar indicate significant differences ($p<0.05$) within each rearing system over time. Small superscript letters over a bar indicate significant difference ($p<0.05$) between rearing systems at the same time point*

Liquid loss

The results of liquid loss in the fillets from RF and PF system showed a significant ($p<0.05$) increase during 10 months storage at -20 °C (Figure 4). The amount of liquid loss increased from (0.2 and 0.15 to 2.3% and 1.5%), in the RF and the PF, respectively. In addition, time to time comparison between RF and the RF did not show any significant differences until 4th month storage, but after 8th and 10th month we observed a significant differences between the

two rearing systems (p<0.05) (Figure 4). In summary, RF showed higher liquid loss compared to PF during the storage time.

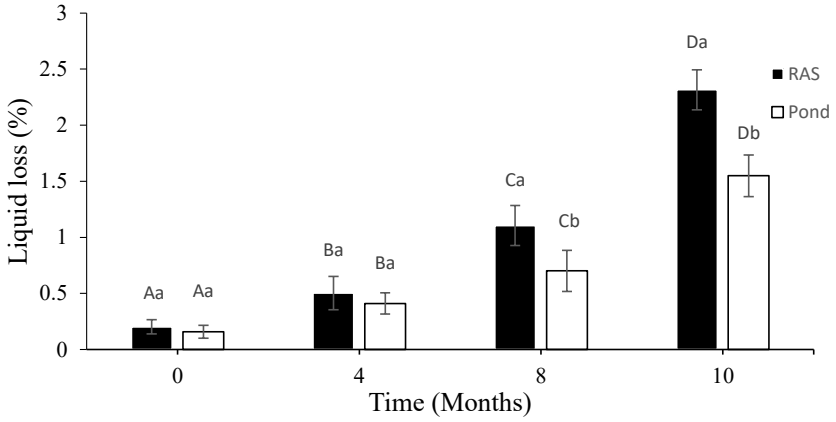

Figure 4. *Changes in liquid loss of perch fillets from RAS and pond system during storage at (−20 °C). Different capital superscript letters over a bar indicate significant differences (p<0.05) within each rearing system over time. Small superscript letters over a bar indicate significant difference (p< 0.05) between rearing systems at the same time point.*

Calpain Activity

The results of calpain activity was measured in the fillet from both rearing systems (RF and PF) (Figure 5). The calpain activity decreased significantly (p< 0.05) after 8 months storage at -20 °C in both rearing systems then it was constant. Furthermore, the comparison of time to time between RF and PF did not show a significant difference in calpain activity during 10 months storage.

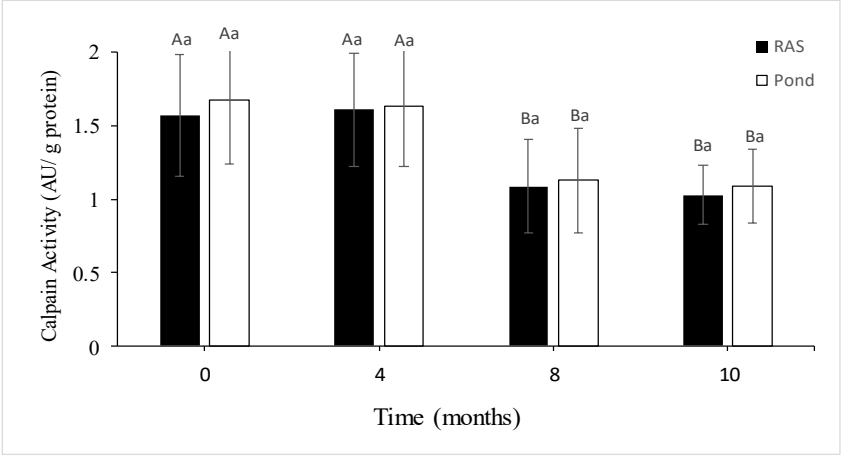

Figure 5. *Calpain specific activity of perch from RF and PF was measured and expressed as relative fluorescent unit per gram protein. Different capital superscript letters above a bar indicate significant differences (p<0.05) within each rearing system. Small superscript letters above a bar indicate significant difference (p<0.05) between rearing systems at the same time point.*

Abbreviation: R= RAS, P= Pond

Colour changes

Table 3. shows the colour changes of the RF and PF during 10 months frozen storage at -20 °C. L* and b* values of the RF and PF increased significantly ($p<0.05$) during the storage time. While a* value in both fillets decreased significantly during the whole period of storage, indicating a more reddish colour. Comparison the results in each time point between the RF and PF showed significant differences in all colour values, which showed more lightness in the PF and more reddish and yellowness in the RF.

Table 3. *Colour changes in fish fillets from RAS and pond during 10 months frozen storage at -20 °C (mean ± S.D., n =6).*

	Time (Months)	RF	PF
Lightness (L*)	0	47.22 ± 2.2^{Aa}	44.26 ± 1.57^{Ab}
	4	48.08 ± 1.89^{Aa}	43.12 ± 1.40^{Ab}
	8	51.55 ± 2.15^{Ba}	45.86 ± 1.39^{Ab}
	10	53.67 ± 1.95^{Ca}	49.10 ± 1.45^{Bb}
Redness (a*)	0	-2.62 ± 0.28^{Aa}	-2.04 ± 0.59^{Ab}
	4	-2.79 ± 0.47^{Aa}	-1.85 ± 0.79^{Ab}
	8	-2.94 ± 0.39^{Ba}	-2.34 ± 0.84^{Bb}
	10	-3.27 ± 0.5^{Ca}	-2.69 ± 0.57^{Cb}
Yellowness (b*)	0	3.29 ± 0.71^{Aa}	5.74 ± 0.78^{Ab}
	4	4.42 ± 1.36^{Ba}	6.43 ± 1.73^{Bb}
	8	4.53 ± 0.76^{Ba}	7.2 ± 1.1^{Cb}
	10	5.03 ± 1.17^{Ca}	7.44 ± 1.07^{Cb}

Different capital superscript letters in a column indicate significant differences ($p<0.05$) within each rearing system. Small superscript letters in a row indicate significant difference ($p<0.05$) between rearing systems at the same time point

Discussion

Stability and proteomic changes of Eurasian perch (*Perca fluviatilis* L.) fillet from two rearing systems have not been reported before. In the previous study we observed a good stability of common carp (*Cyprinus carpio* L.) fillet during 6 months storage at -20°C (Hematyar, et al., 2018). Due to the fact that carp is a moderate fatty fish (8%) and perch is a lean fish (1%) we consider longer storage time (10 months) in this study in order to investigate more deeply on the progress of lipid and protein oxidation. For further investigation, we used proteomic to find the protein pattern in RF and PF. SDS-PAGE showed more fainted bonds in the PF compared to the RF. In contrast, Addis et al. (2010) could not observe any differences in the total protein profiles, when compared farmed and wild sea breams fillets. However, declined intensity in actin and myosin bands were very pronounced in the fillets of both rearing systems with respect to elapsed time. Actin and myosin are the most prone proteins to oxidise in the muscle foods (McDonagh, Tyther, & Sheehan, 2005). Sharp & Offer (1992) reported that 80% of myosin in the cod fillets is deteriorated in the frozen storage compared to the fresh myosin. This study confirmed the protein denaturation in the mentioned bands specially, after 8 and 10 months storage. Additionally, higher intensity of MHC and actin till 4 months storage in PF than RF indicating fragmentation of the other higher molecular weight proteins. Furthermore, RF showed higher amount of liquid loss and firmness compared to the PF in each time point. In line with this results Martelli et al. (2013) reported a higher liquid loss in the meagre

(*Argyrosomus regius*) fillets which were reared in a tank system compared to the meagre fish fillets from cage system. The denaturation of nebulin, troponin, and serum albumin indicating the deterioration of fillet texture in both systems. With respect to SDS-PAGE result fainted nebulin and troponin bands as well as high reduction in MHC and actin intensity in PF rather than RF might be a reason for lower firmness and WHC.

The immunoblot indicated that proteins with size around 40 to 43 kDa were oxidized heavily. In line with our results Baron, et al. (2007); Eymard, Baron, & Jacobsen (2009) reported oxidized protein carbonyl group at high and low molecular weight in the fillets of rainbow trout (*Oncorhynchus mykiss*) and horse mackerel (*Trachurus trachurus*) during the storage time at -20 °C and +5 °C. The result of our study with respect to the storage time showed higher amount of protein oxidised carbonyl after 8 and 10 months in both fillets. On the other hand, more fainted bands in PF and more oxidised carbonyls in RF especially, at time 0 and 4 months storage indicate that probably, the structure of proteins and peptides in both fillets might be differ. Dalsgaard, Otzen, Nielsen, & Larsen (2007) reported that random coiled proteins compared to the globular proteins are more prone to oxidation. In case of our result probably, PF holds a more tight and well-define structure than RF had more random coiled proteins (secondary). Due to the structures, globular proteins are more protected against oxidation during the storage time (Eymard, et al., 2009). In addition, immunoblotting is not a quantitative method, may be fewer proteins were oxidised in the RF, but they were oxidised more heavily compared to the PF. Furthermore, we observed a major reduction in firmness after 4 months storage in PF and RF (43 and 40%) respectively while in the reminding times we could not observe this percentage. According to immunoblot result less oxidised carbonyl in t=0 and t=4 have been reported than t=8 and t=10 but higher firmness reduction reported in the first 4 months of storage. It appears that, formation of ice crystal follow by cell disruption can be considered as a key role rather than protein denaturation during first months of frozen storage while in the rest of storage time protein denaturation is dominated. On the other hand, considering the last month of storage (t=10) showed significantly higher firmness and liquid loss in the RF rather than PF due to the higher aggregated and cross-linked proteins. Additionally, decreasing trend in calpain activity from the fillets of both rearing systems, also confirmed the mentioned above statement about the role of ice crystal in the deterioration of fish fillet. The calpain activity trend has been reported by Nagaraj & Santhanam (2006) in the goat muscles during 120 days frozen (-15 °C) storage. Moreover, PF had higher but not significantly calpain activity rather than RF which is in line with our firmness result. Higher oxidation and cross-linked proteins might be responsible for lower calpain activity in RF compared to PF (Lametsch, Lonergan, & Huff-Lonergan, 2008).

Native page result, showed less intensity in the protein bands in PF specially, after 8 and 10 months storage rather than RF which is in line with our SDS-PAGE result. It has been reported by Lene Engelsgjerd Kramer (2013) that the loss of protein band at around 480 kDa might be related to the myosin complex, while the stable band at around 242 kDa indicates myosin heavy chain. Fainted protein bands between 480-720 kDa showed the denaturation of myosin in the PF while in the RF was stable. Moreover, less intensity in some high molecular protein bands in PF confirm more fragmentation compared to RF which might be related to higher bands intensity in PF with respect to SDS-PAGE result. The structure of native protein can be stabilised by sulphide linkages. This results showed more disulphide bonds in the RF compared to the PF which confirm more cross-linked proteins in the RF.

The present study showed the content of protein carbonyls in RF extracts were significantly higher than PF at t=0 and 4 by UV spectrophotometric method while, amount of MDA did not show any significant differences between both fillets. It is remarkable that the significant progress in the amount of protein carbonyl measured by spectroscopy after 10 months

storage at -20°C in both fillets was clearly visible in our immunoblotting result. Furthermore, higher levels of oxidised protein in RF than PF indicating that stunning induced more free radical attack in RF and presence antioxidants could not to cope with it. This might be related to the higher consumption of natural antioxidants in the fish were reared in pond system. Moreover, we observed a high and positive correlation between MDA and protein carbonyl content (r=0.98 and r=0.97) in the RF and PF respectively. Probably, interaction between lipid and protein oxidation products in both fillets can be considered for high oxidation progress. Additionally, the products of lipid and protein oxidation considerably increased after 4 months storage in fillets from both rearing systems which confirm that probably, lipid and protein oxidation started together in RF and PF during frozen storage, which has been recently indicated (Hematyar, et al., 2019).

On the other hand, carbonyls and MDA value enhanced significantly in the both fillets during 10 months storage at -20 °C. However, in case of MDA did not reached to the critical amount (1.5 mg MDA kg^{-1}) (Ke, et al., 1984). This increasing trend in amount of lipid and protein oxidation products have been reported before by Baron, et al. (2007); Dulavik, et al. (1998) in rainbow trout (*Oncorhynchus mykiss*) and asinsaithe (*Pollachius virens* L.) fillets. Formation of ice crystal during frozen storage can release heme-iron pro-oxidants and also, leads to partial dehydration of proteins, which can increase lipid oxidation and protein denaturation in fish muscle, respectively (Leelapongwattana, et al., 2005). Also, higher L* parameter in the RF compared to PF might be related to the higher level of secondary lipid oxidation products.

Reznick & Packer (1994) reported a relationship between average protein molecular mass and amount of protein carbonyl confirm that 50 kDa is linked to 2 nmol protein carbonyl with percent of 10% carbonyl relative to protein. According to our result the amount of carbonyl reached to 3.1 nmol (15%) after 4 and 8 months in RF and PF respectively indicates the contribution of 50% myosin, nebulin and some high molecular weight protein like titin. Regarding this statement we observed more intense myosin, nebulin, actin and higher molecular weight proteins band in PF compared than RF which indicates less denaturation as well as more stability against oxidation in PF.

According to our results, PF illustrated more, darker and reddish while RF showed more white and light colour. The difference in colour was visually distinguishable. In line, fish reared in the pond system showed higher a* value than RAS which indicate more redness. This might be related to the consumption of zooplankton and zoobenthos in the pond system which may increase the redness of fish flesh (Winfield, 2001).

Finally, we conclude that probably lipid and protein oxidation started together in our study. Furthermore, during first months of frozen storage formation of ice crystal can be considered as the main factor for the development oxidation which has negative effect on textural parameters as well. Higher lipid and protein oxidation in RF might be related to different protein structures.

Conclusion

This study indicated the impact of rearing conditions on the fish fillet quality and the development of lipid and protein oxidation during long term storage at -20 °C. Both lipid and protein oxidation products were lower in PF compared to the RF. On the other hand, RF showed higher liquid loss and firmness than PF indicating more aggregated and cross-linkage proteins. Concerning the proteomic analysis (more fainted bands and less oxidised carbonyl in PF compared to RF till t=4), amount of carbonyl and firmness we may conclude that until 4 months storage formation of ice crystal follow by cell disruption are the main issue for deterioration of PF while in RF protein denaturation is the main reason. It seems, optimizing the amount of antioxidants in the artificial feeds can be investigate more deeply for the future studies.

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CHAPTER 5

POST- MORTEM QUALITY CHANGES OF EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FILLETS FORM TWO DIFFERENT REARING SYSTEMS

Hematyar, N., Stejskal, v., Sampels, S., Prokesova, M., Matoušek, J., Vacha, F., Krizek, M., Dadakova, E., Mraz, J., Post- mortem quality changes of Eurasian perch (*Perca fluviatilis* L.) fillet form two different rearing systems. Manuscript

My share on this work was 50 %.

POST- MORTEM QUALITY CHANGES OF EURASIAN PERCH (*PERCA FLUVIATILIS* L.) FORM TWO DIFFERENT REARING SYSTEMS

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Abstract

Lipid and protein oxidation, as well as their relation to chosen quality parameters in Eurasian perch fillets from recirculating aquaculture system (RAS) and pond systems were assessed during 12 days storage at +3.5°C. Lipid and protein oxidation increased significantly ($p < 0.05$) during the storage irrespective of aquaculture system. However, there was no statistical difference in MDA values between fillets from different rearing system over time. The carbonyl concentration was significantly high in RAS originate fish fillets in comparison to fillets from RAS fish. Firmness of pond fillets dropped significantly (until the 8th day) while RAS fillets demonstrated some consistency in firmness. Drip loss increased significantly in fillets over the storage time irrespective of system origin. However, from the magnitude of drip loss was higher in RAS fillets. No significant differences were observed in pH and onset of rigor mortis in fillets from either systems. In terms of appearance (colour), RAS fillets demonstrated significant increase in C*ab and decrease in H* value. Pond origin fillets showed an overall significant deterioration in colour properties (L*, b* and H* values). For Biogenic amines, fillets from either rearing system were presumed safe due low histamine levels. The differences being non-significant in most cases, except few exceptions like carbonyl concentration (higher in RAS fish), firmness (lower in pond fish), drip loss (lower in pond fish) and colour properties (better in RAS fish). Biogenic amine concentrations were far below critical concentration.

Keywords: *Carbonyls, MDA, rigor index, firmness, Drip loss*

1. Introduction

Fish provide a high amount of the highly unsaturated fatty acids especially eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) and the beneficial effects of the consumption of these fatty acids on human health have been reported (Ugoala, Ndukwe, & O Audu, 2008). Fillet of Eurasian perch (*Perca fluviatilis* L.*)* is a highly regarded product and has a high price in the market (Watson, 2008). The main production of perch is still traditional pond polyculture or wild catches. During the past two decades intensive methods utilising recirculating aquaculture systems (RAS) have been expanded for the variation of European inland aquaculture (Fontaine, 2009). In this system rearing conditions such as high stocking density (up to 60 kg m-3), stable temperature (23°C), uniform photoperiod (12L:12D) and also commercially formulated feeds are applied (Fiogbé & Kestemont, 2003). Stocking density is an important parameter in order to evaluate the profitability and productivity of commercial fish farms (Mairesse, Thomas, Gardeur, & Brun-Bellut, 2007). Farming conditions can influence some parameters in fish fillet, namely muscle fiber development, pigment composition and biochemical composition which can decisively change the colour and texture in the fillet. Formulated feeds can change flesh quality and have an impact on market value (Mairesse et al., 2007). Fish reared in RAS has a better grow rate as a result of enough feed, optimal temperature or suppressed maturity. On the other hand, in pond fish reared with fluctuated temperatures have different growth rates and they also feed on more natural food like zooplankton and zoobenthos, which are responsible for colour changes in fish fillet. Besides feed composition, water temperature and stock density are different in ponds and RAS which can also affect the fillet quality (Sveinsdottir et al., 2009). Fish in ponds also undergo maturity process which is affecting fillet quality.

Freshness can be considered as the most critical attribute when fish quality is evaluated (Gutérrez Guzmán, Fernández Segovia, Fuentes López, Ruiz Rico, & Barat Baviera, 2015). Due to the lipid oxidation, autolysis and microbial activity fish is very perishable food (Parlapani, Mallouchos, Haroutounian, & Boziaris, 2014). When fish is slaughtered, some post mortem changes can take place. These modifications such as increased microbial growth, decrease of pH, changes in firmness, increased drip loss, changes in colour and lipid- and protein oxidation are affect fish fillet quality and shelf life during the storage time (Ehira & Uchiyama, 1987). Microbial deterioration has a key role that can negatively impact on the quality and freshness of fish fillet during the storage time. Refrigerated storage is widely used before technological processes or consumption for the protection of fish quality. Even during refrigerated storage, the spoilage microorganism's growth, the enzymatic activity and the chemical are slower, but still over time they lead to diminished fish quality (Cakli, Kilinc, Cadun, Dincer, & Tolasa, 2007).

A review of the available literature revealed a lack of comprehensive research on post mortem changes, especially biogenic amines (BAs) formation. Biogenic amines are formed by decomposition of the proteins, and their amount can be used as an additional criterion for the microbial degradation of the fish. In this respect, especially diamines such as putrescine (PUT), cadaverine (CAD) and tyramine (TYM) are important. The first signs of deterioration of organoleptic parameters of fish flesh usually occur when PUT or TYM contents exceed 10 mg/kg or CAD 15 mg/kg (Chytiri et al., 2004; Li et al., 2012). Histamine (HIM) has been widely studied in the literature for similar reasons, but most importantly, it is primarily a toxic substance (Prester, 2011). Polyamines spermidine (SPD) and spermine (SPM) are secondary products of putrescine transformations. These substances are also sometimes included among the criteria characterizing degradation processes. With emerging of new progressive methods for the detection of BAs (Gil et al., 2008; Toniolo et al., 2014), will be their use as flesh qualitative markers greatly facilitated. Beside the microbial spoilage, which is considered

as an important shelf life factor, a high proportion of unsaturated fatty acids, also makes the fillets prone to oxidation during the storage time. Finally, as the result of the lipid oxidation some volatile compounds are generated which are responsible for unpleasant odour during the storage. Additionally, also fish proteins are susceptible to oxidation which can lead to undesirable changes of firmness, and water holding capacity (WHC) (Baron, Kjaersgard, Jessen, & Jacobsen, 2007). Moreover, several authors have reported the interaction between the secondary products of lipid oxidation with proteins during the storage time, which can enhance the progress of initial oxidation reactions (Garcia & Cava, 2004).

The aim of this work was to study, compare and explore inter-relationships among (a) development of lipid and protein oxidation, (b) common organoleptic properties like firmness and appearance, (c) biogenic amine concentrations and associated risks in Eurasian perch fillets from two different aquaculture systems (extensive pond-based culture and intensive RAS culture), and, (d) dynamics of rigor-mortis during refrigerated storage.

Abbreviations: Fillet from RAS (RF), Fillet from pond (PF)

2. Materials and Methods

2.1. Experimental design

Forty eight market size Eurasian perch (average 183 g and 211 g for (n=30 fish) in weight for RAS and pond fish respectively) with the pond age 2 + and RAS age 1+, from two different rearing systems (traditional pond culture and RAS) were used in the present study.

Pond reared perch were obtained from the fish farm Rybarstvi Nove Hrady Inc. (pond Zar) located in the south of the Czech Republic (48°8′N, 14°7′E). Fish were reared in polyculture (natural production of 240 kg ha^{-1}) with common carp (*Cyprinus carpio*) as a main fish species. The food source was natural prey with the main forage fish being top mouth gudgeon (*Pseudorasbora parva*), roach (*Rutilus rutilus*) and benthic organisms. The fish were harvested on 19 October during the common Czech harvest period.

Fish from RAS were reared at the faculty premises and fed commercial feed (Inicio plus, BioMar, France) with the nutrient values: protein 52%, fat 23%, fibre 0.9%, carbohydrates (NFE) 12%, gross energy 23.5 MJ kg^{-1}, digestible energy 20.6 MJ kg^{-1} (manufacturer's data).

Fish were kept on tanks at the faculty at the same temperature (20°C) for one week before being slaughtered, gutted, skinned, washed and filleted on the faculty premises by one trained person. Fillets from fish were randomized packed in plastic bags, labelled and stored in a refrigerator at +3.5°C. Firmness and chemical analyses were executed on the first day and after 4, 8 and 12 days of storage.

2.2. pH

The pH of each fish fillet was measured with inserting a pH probe (Testo 206, Lenzkirch, Germany) into the upper mass of the fillet, just behind the head.

2.3. Rigor index

The rigor mortis development was measured by Cuttingers method (tail drop) (Bito, 1983). The rigor index (Ir) was calculated by the formula $Ir = [(Lo-Lt)/Lo] \times 100$, where L represents the vertical drop (cm) of the tail when half of the fish fork length is placed on the edge of a table as a function of time. The tail drop at the beginning of the experiment is Lo, while

Lt represents measurements throughout the experiment (t = 0-142 hours with interval of 3 hours).

2.4. Fatty acids profile

Lipid analyses were performed as described by (Mráz & Pickova, 2008). Lipid extraction was done according to (Hara & Radin, 1978). First 1 gram of sample was weighed. Then, samples were homogenised with 10 ml HIP and finally, vortex it well with 1 ml hexane. Transfer the upper phase to the pre-weight tube and evaporate the combined hexane phase and weigh the tube, calculate the fat content.

Methylation was executed with BF3 according to (Appelqvist, 1968) and analyses by GC were performed following the procedures described earlier by (Sampels, 2015).

2.5. Thiobarbituric acid reactive substances (TBARS)

The analysis of TBARS was conducted according to Miller (1998). The semi frozen samples were minced, connective tissues and visible fat were removed, and a subsample of approximately 1 g of muscle tissue was taken for analysis. The samples were homogenized with 9.1 mL (0.61 mol/L) of trichloroacetic acid (TCA) solution and 0.2 mL (0.09mol/L) of butylated hydroxytoluene in methanol, using an UltraTurraxT25 (IKA-Labortechnik, Janke & Kunkel, Staufen, Germany) for 3–20 s at a speed of approximately 14 000 rpm. Then, the homogenate was filtered through a Munktell paper (Munktell Filter AB, Grycksbo, Sweden). The filtrate (1.5 mL) was transferred twice to two new tubes; then, 1.5 mL of thiobarbituric acid (TBA) solution (0.02 mol/L) was added to the first tube (test sample), and 1.5 mL water was added to the second tube (sample blank). After reaction in darkness overnight at room temperature (20°C), the reaction complex was analysed at a wavelength of 530 nm against the sample blank using a UV-visual spectrophotometer (Specord 210; Analytik Jena, Germany).

2.6. Sample extraction and derivatisation

Samples were homogenised using an Ultra-Turrax T25 homogeniser (Ika Labortechnik, Staufen, Germany). Biogenic amines were extracted from homogenised material with diluted perchloric acid, p.a. (0.6 M). After filtration the volume was made up to 150 ml with per chloric acid. The amines were determined as dansyl derivatives after derivatisation with dansyl chloride by UPLC. The procedure has been described in detail by (Dadakova, Krizek, & Pelikanova, 2009). Quality index (QI) and Biogenic amines index (BAI) was calculated using followed formulae (contents of BAs are given in $mg.kg^{-1}$):

QI= (histamine + putrescine + cadaverine)/ (1+ spermine + spermidine)

BAI=putrescine + cadaverine +histamine + tyramine

2.7. Protein oxidation (DNPH)

Protein oxidation was estimated as carbonyls content after incubation with 2,4 dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid following a slightly modified method described by (Levine et al., 1990). 0.5–1 gram of meat will was homogenized in 10 ml KCl (0.15 M) using an UltraTurrax (Janke & Kunkel, Staufen, Germany, T25IKA-Labortechnik,) for 3 × 20 s at a speed of approximately 14000 rpm. 100 µl of homogenate solution was pipetted

into 2 ml centrifuge tubes (Eppendorf) and 1ml trichloroacetic acid 10% (TCA) was added to each tube then, centrifuged at 5000 rpm for 5 min. The supernatant was removed. 1ml HCl (2N) to the blank and 1ml 2,4 dinitrophenylhydrazine in 2 M HC1 were added and allowed to stand at room temperature for 1 hour, with vortexing every 20 min. Then 1 ml trichloroacetic acid (10%) was added and centrifuged the tubes in a table top micro centrifuge (5000 rpm) for 5 min, and the supernatant was discarded. The pellets were centrifuged with 1 ml ethanol-ethyl acetate (1:1) in 10000 rpm for 5 min. The pellets were washed 2 times with 1 ml ethanol-ethyl acetate (1:1) to remove free reagent. The precipitated protein was redissolved in 1.5 ml guanidine solution (6M) and centrifuged in the micro centrifuge for 2 min at 5000 rpm. The spectrum, read against the complementary blank in the case of cruder samples or against water in the case of purified proteins. The carbonyl concentration was analysed as DNPH calculated on the basis of absorption of 21.0 mM^{-1} cm^{-1} at 370 nm for protein hydrazine. Protein concentration was measured at 280 nm in the same sample and quantified by using bovine serum albumin as a standard.

2.8. Fillet colour

Fillet colour was evaluated by the Minolta Colorimeter (Spectro Photo Meter, CM- 600d, Konica, Japan) as described by (Folkestad et al., 2008). The parameters L* (lightness), a* (red-green spectrum), and b* (yellow-blue spectrum) were used to study the colour properties of the fillet surface, where L* is the luminance score and ranges from zero (black) to 100 (white) and a* and b* are chromatic scores. The hue (H*) and chroma (C*) values were calculated from the b* and a* values. Hue is the visible colour and is expressed by the equation, H*= tan-1(b*/a*) (Jha, 2010). The chroma value is an expression of the saturation of the colour. It is calculated by the equation; C*= (a*2+b*2)1/2 (Jha, 2010).

2.9. Firmness analysis

Firmness analysis was performed instrumentally using a firmness analyser (TA-XT. Plus, Stable Micro systems UK) by pressing a flat-ended cylinder (10 mm diameter, type P/10) into the section of fillet below the dorsal fin perpendicular to the muscle fibers at a speed of 2 mm s^{-1} until the fillet was compressed to 50% of its original thickness. Firmness was defined as the maximum force detected during first compression, expressed in grams.

2.10. Drip loss

Drip loss of perch fillets was measured according to the (Duun & Rustad, 2008) method with slight modifications. The fillets were removed and the liquid left in the bag was collected and weighed. Drip loss was expressed as g drip liquid/ 100 g muscle.

2.11. Statistical analysis

Statistical evaluation was done by using one-factor ANOVA analysis in the Statistica CZ 12 software package. The level of significance was considered at ($P<0.05$) and the results are presented as mean ± S.D.).

3. Results

3.1. pH

Results of pH did not show any statistical changes during 140 hours storage of RF and PF at +3.5ºC ($p<0.05$). Comparing time to time pH, between RF and PF showed no statistical changes until 60 hours storage but from 72 hours to 140 hours there were significant differences ($p<0.05$) (Figure 1).

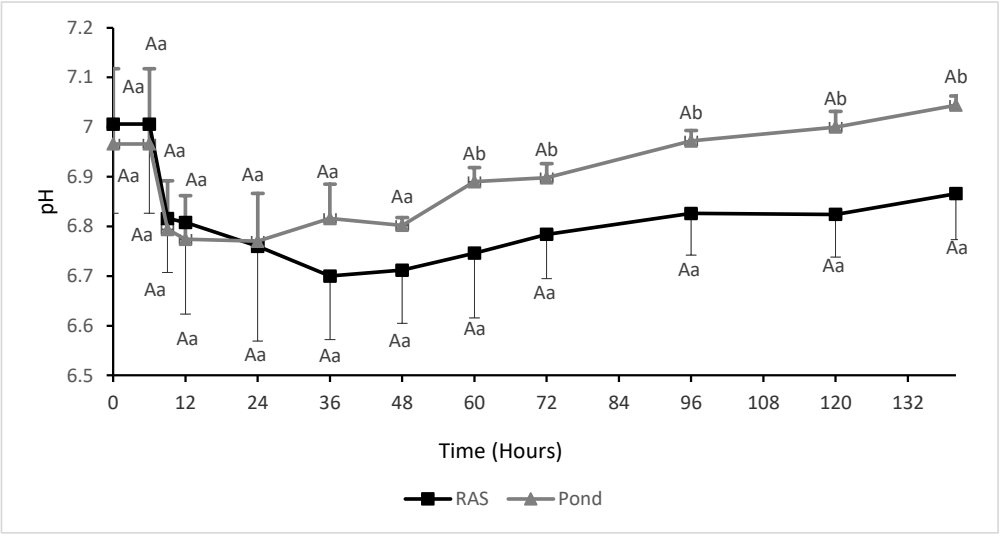

Figure 1. *pH of fish fillets from RAS and pond during 140 hours refrigerated storage at +3.5ºC (mean ± S.D., n=6). Different capital superscript letters over a time point indicate significant differences ($p<0.05$) within each rearing system over time. Small superscript letters over a time point indicate significant difference between rearing systems at the same time point.*

3.2. Rigor index

The results of rigor index are shown in (Figure 2). Rigor index was measured during 142 hours keeping fish at +3.5ºC. Onset of rigor was faster in the RF compared to the PF but not significantly ($p<0.05$). In the RF and PF post-mortem changes started at 6 and 8 h, respectively.

In addition, there was a significant difference between maximum rigor index in both systems ($p<0.05$). Full rigor in the RF and PF was observed after 12 and 24 h respectively.

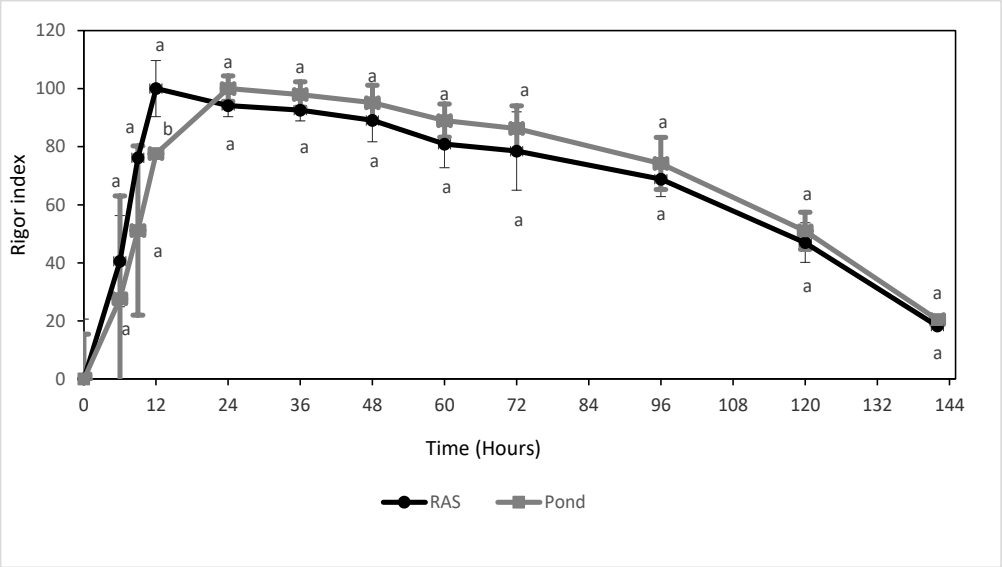

Figure 2. *Changes of rigor index of fish fillets from RAS and pond during 140 hours refrigerated storage at +3.5°C (mean ± S.D., n=6). Different small superscript letters over a time point indicate significant difference (p<0.05) between rearing systems at the same time point.*

3.3. Fat content and fatty acids profile

Fat content in the fillet from both systems did not show any significant difference ($p<0.05$) (Table 1). The proportions of SFA, MUFA and PUFA in the fresh RF and PF were 25.85%, 30.85%, 43.3% and 25.72, 26.56 and 47.72 respectively (Table 1). Comparison between SFA, MUFA and PUFA in both fillets did not show any statistically changes in the amount of SFA while, MUFA was significantly higher in the RF and PUFA was significantly higher in the PF.

Total amount of saturated fatty acids (SFA) were the same in both groups. Palmitic acid (C16:0) showed the major SFA, but there was no statistically changes between both systems. Myristic acid (C14:0) and stearic acid (C18:0) were also present at high levels. RF showed significantly higher amount of C14:0 and PF had significantly higher amount of C18:0. However, the amount of others C20:0 (Eicosanoic acid), C22:0 (Docosanoic acid) and C24:0 (Tetracosanoic acid) were low or no detectable in both fillets.

Regarding to the MUFA, results showed a significant differences between the fillets both groups. RF showed significantly higher amount of C14:1 (Myristoleic acid), C18:1n-9 (Oleic acid) and C20:1n-9 (gondoic acid) fatty acids and PF showed significantly higher amount of C16:1 (palmitoleic acid) and C18:1n-7 (vaccenic acid). Furthermore, there was no significant changes observed in the amount of C22:1(erucic acid) and C24:1(nervonic acid) fatty acids.

Total amount of PUFA showed significant difference between fillets from both systems. RF showed significantly higher amount of linoleic (LA, 18:2 n-6) and docosahexaenoic acid (DHA, C22:6 n-3). However PF showed significantly higher amount of alpha-linoleic acid (aLA, 18:3 n-3), docosapentaenoic acid (C22:5 n-3) arachidonic acid (AA) (20:4 n-6), eicosatrienoic acid (ETE) (20:3 n-3) and eicosapentaenoic acid (EPA, Timnodonic acid) (20:5 n-3). The amount of eicosadienoic acid (20:2 n-6) was the same in both systems.

There was also no difference between groups in Σn-3:Σn-6 ratio which means that the ratio was not influenced by the culture method.

Table. 1. *Total fat content (%) and fatty acid composition (%, of total identified) in fresh fillets from RAS and pond.*

	RAS	Pond
Fat content	1.19±0.23a	1.09±0.31a
Fatty acid	**RAS**	**Pond**
C14:0	2.14±0.22a	1.62±0.11b
C16:0	20.8±0.47	20.38±0.79
C18:0	2.8±0.33a	3.63±0.41b
SFA	**25.85a**	**25.72a**
C14:1	0.24±0.04a	0.14±0.03b
C16:1	6.53±0.39a	8.15±1.43b
C18:1n-7	2.68±0.21a	4.47±0.38b
C18:1n-9	19.26±1.11a	13.38±1.58b
C20:1n-9	1.78±0.21a	0.36±0.06b
MUFA	**30.85a**	**26.56b**
C18:2n-6	7.19±0.52a	3.96±0.55b
C18:3n-3	1.32±0.11a	4.37±0.20b
C20:4n-6	1.29±0.14a	7.12±0.95b
C20:5n-3	4.89±0.38a	6.52±0.70b
C22:6n-3	26.95±1.77a	22.18±1.04b
C22:5n-3	1.08±0.10a	2.66±0.13b
PUFA	**43.2a**	**47.62b**
n-3	34.4±11.36a	29.99±8.64a
n-6	8.8±3.71a	11.41±3.39a
n-3/n-6	3.90a	2.62a

Small superscript letters in a row indicate significant difference ($p<0.05$) between rearing systems. Abbreviation: ND = not defined

3.4. Biogenic amines composition

It is not surprising that the contents of biogenic amines (BAs) were significantly ($p<0.05$) lower for the flesh stored at a lower temperature (+ 3.5°C) in both systems compared to the higher temperature (+12°C) (Table 2). Also in the initial stages of storage there was a statistically significant difference between the RF and PF. This is particularly evident during the first 14 days in case of PUT and TYM changes. On the other hand, this trend was not observed in CAD. In the RF, the PUT and TYM contents were always lower during the first 14 days. After 14 days of storage the differences in both breeding variants were not statistically significant, probably due to the more advanced degradation of the protein matrix. It is also important that HIM was not detected at this temperature during the first 14 days of storage. At higher temperature (12°C), the PUT and TYM content increased very dramatically and already on the 7th day their content exceeded ten times the common content for high quality fish flesh. At this temperature (12°C), there were statistically significant differences between flesh from intensive culture (RAS) and pond. This difference was evident in the first two weeks of storage. In the case of RAS group, the contents of both amines (PUT and TYM) were again lower.

Table 2. *Content of putrescine, cadaverine and tyramine (mg/kg) in fillets of Eurasian perch stored at 3.5 and 12°C.*

	Time (days)	7	14	21	28
Putrescine					
RAS	(3.5°C)	ND^{A}	4.6 ± 2.30^{A}	26.3 ± 6.15^{A}	51.6 ± 15.6^{A}
PON	(3.5°C)	1.7 ± 0.48^{B}	10.6 ± 2.89^{B}	33.2 ± 2.23^{A}	41.7 ± 14.3^{A}
RAS	(12°C)	117 ± 21^{C}	355 ± 37^{C}	824 ± 92^{B}	-
PON	(12°C)	196 ± 28^{D}	512 ± 43^{D}	958 ± 105^{B}	-
Cadaverine					
RAS	(3.5°C)	ND^{A}	2.3 ± 0.15^{A}	17.6 ± 9.56^{A}	27.9 ± 14.8^{A}
PON	(3.5°C)	ND^{A}	2.4 ± 0.66^{A}	9.1 ± 2.58^{A}	13.8 ± 10.6^{A}
RAS	(12°C)	90.2 ± 33^{B}	346 ± 71^{B}	774 ± 151^{B}	-
PON	(12°C)	281 ± 42^{C}	550 ± 65^{C}	606 ± 137^{B}	-
Tyramine					
RAS	(3.5°C)	1.4 ± 0.21^{A}	1.7 ± 0.72^{A}	8.8 ± 3.13^{A}	16.4 ± 6.10^{A}
PON	(3.5°C)	2.3 ± 0.57^{B}	3.9 ± 0.80^{B}	12.5 ± 4.68^{A}	16.7 ± 5.15^{A}
RAS	(12°C)	39.8 ± 18^{C}	236 ± 77^{C}	595 ± 81^{B}	-
PON	(12°C)	107 ± 14^{D}	377 ± 41^{D}	522 ± 92^{B}	-
Histamine					
RAS	(3.5°C)	ND^{A}	ND^{A}	ND^{A}	0.72 ± 0.45^{A}
PON	(3.5°C)	ND^{A}	ND^{A}	ND^{A}	0.68 ± 0.17^{A}
RAS	(12°C)	18.6 ± 11^{B}	89.8 ± 31^{B}	229 ± 101^{B}	-
PON	(12°C)	465 ± 128^{C}	516 ± 124^{C}	312 ± 118^{B}	-
Spermidine					
RAS	(3.5°C)	2.2 ± 0.38^{A}	2.4 ± 0.52^{A}	2.2 ± 0.17^{A}	1.8 ± 0.23^{A}
PON	(3.5°C)	3.0 ± 0.66^{A}	3.2 ± 0.30^{A}	2.4 ± 0.89^{A}	2.0 ± 0.36^{A}
RAS	(12°C)	2.5 ± 0.65^{A}	1.0 ± 0.63^{B}	ND^{B}	-
PON	(12°C)	1.3 ± 1.17^{A}	ND^{C}	ND^{B}	-
Spermine					
RAS	(3.5°C)	6.6 ± 1.04^{A}	7.1 ± 0.78^{A}	7.2 ± 0.68^{A}	6.1 ± 1.23^{A}
PON	(3.5°C)	4.8 ± 0.36^{B}	7.0 ± 0.41^{A}	4.2 ± 0.49^{B}	4.4 ± 1.21^{A}
RAS	(12°C)	6.4 ± 0,77^{A}	3.5 ± 1.34^{B}	ND^{C}	-
PON	(12°C)	3.2 ± 0,51^{D}	0.5 ± 0.28^{C}	ND^{C}	-

Small superscript letters in a row indicate significant difference ($p<0.05$) between rearing systems.
Means indicated by different capital letters in the same column differ significantly ($P<0.05$).
Abbreviation: Recirculating aquaculture systems (RAS), Extensive pond-based (PON) system.

Abbreviation: ND = not defined.

3.5. Lipid and protein oxidation

The MDA values in the fillets from the fish reared in two different conditions (RAS and pond) are shown in (Table 3). In fillets from both rearing systems MDA increased significantly ($p < 0.05$) during the 12 days of refrigerated storage (+3.5°C). The MDA value was 0.28 MDA (µg/g) on the first day and enhanced to 0.36 and 0.32 MDA (µg/g) in RF and the PF respectively. Furthermore, during the 12 days storage there were no statistical differences between RF and

PF. The initial amount of MDA was the same in fish fillet from both systems but during the storage time, the MDA value increased more in RF compared to those from PF.

The carbonyl contents increased significantly during the storage time in RR and PF ($p < 0.05$) (Table 3). The initial amount of carbonyls was 1.90 and 0.47 (nmol/mg), which increased to 3.01and 1.28 (nmol/mg) in RF and PF respectively after 12 days. The carbonyl content in PF was significantly lower than in RF at all time points.

Table 3. *MDA (µg/g) and Carbonyl content (nmol/mg) parameters in perch fillets from RAS and pond during refrigerated storage at +3.5°C*

	MDA (µg/g)		**Carbonyls (nmol/mg)**	
Time (Days)	RAS	Pond	RAS	Pond
0	0.28 ±0.01^{Aa}	0.28 ±0.0^{Aa}	1.9 ±0.01^{Aa}	0.47 ±0.00^{Ab}
4	0.29 ±0.01^{Aa}	0.28 ±0.02^{Aa}	2.24 ±0.01^{Aa}	0.53 ±0.02^{Ab}
8	0.34 ±0.05^{ABa}	0.31 ±0.02^{ABa}	2.71 ±0.05^{Ba}	0.9 ±0.02^{Bb}
12	0.36 ±0.04^{Ba}	0.32 ±0.02^{Ba}	3.01 ±0.04^{Ca}	1.28 ±0.02^{Bb}

Different capital superscript letters a column indicate significant differences ($p<0.05$) in each rearing system. Small superscript letters a column indicate significant difference ($p<0.05$) between rearing systems at the same time point.

3.6. Colour changes

Table 4 shows the changes of colour of the fillets during storage time. L* values for the PF decreased significantly from the first day until the 8th day ($p<0.05$) but in RF there were no statistical changes, indicating that the PF changed more compared to RF. While a* values in the RF remained similar, they increased slightly (not significantly) in PF, showing a more reddish colour. In PF the b* value was significantly decreased on the 4th day ($p<0.05$) while values on day 8 and 12 were intermediate. The chroma value in RF increased significantly but did not change significantly in PF. The hue values decreased significantly in RF during the storage time while, they decreased significantly in PF system from the 4th day until the 12th day. L*, a*, b*, C*ab and H* parameters differed significantly between fillets from the different systems at all time points except for a*, C*ab and H* values on the 8th day.

Table 4. *Colour changes in fish fillets from RAS and pond during refrigerated storage at +3.5°C*

	Time (Days)	**RAS**	**Pond**
Lightness (L*)	0	47.23±2.1^{Aa}	44.26±2.16^{Ab}
	4	46.2±1.69^{Aa}	43.19±2.89^{ABb}
	8	46.64±1.49^{Aa}	40.99±2.34^{Bb}
	12	46.66±4.54^{Aa}	42.45±2.25^{ABb}
Redness (a*)	0	-2.61±0.28^{Aa}	-2.04±0.59^{Ab}
	4	-2.63±0.47^{Aa}	-2.63±0.79^{Ab}
	8	-2.3±0.39^{Aa}	-1.96±0.84^{Aa}
	12	-2.69±0.5^{Aa}	-1.89±0.57^{Ab}

Yellowness (b*)	0	2.00±1.55^{Aa}	5.74±1.59^{Ab}
	4	2.46±1.28^{Aa}	3.78±0.86^{Bb}
	8	2.54±0.99^{Aa}	4.49±1.25^{ABb}
	12	3.45±1.17^{Aa}	5.04±1.07^{ABb}
C^*_{ab}	0	5.80±0.74^{Aa}	16.99±6.75^{Ab}
	4	6.23±1.79^{ABa}	11.59±2.90^{Ab}
	8	6.34±0.9^{ABa}	11.96±4.93^{Aa}
	12	9.98±1.31^{Ba}	14.49±2.28^{Ab}
H*	0	143.88±1.88^{Aa}	114.46±8.42^{ABb}
	4	140.97±7.29^{ABa}	126.24±5.61^{Ab}
	8	129.44±2.60^{ABa}	116.85±13.64^{ABa}
	12	128.37±7.39^{Ba}	113.30±5.55^{Bb}

Different capital superscript letters in a column indicate significant differences ($p<0.05$) within each rearing system. Small superscript letters in a row indicate significant difference ($p<0.05$) between rearing systems at the same time point.

3.7. Firmness changes

The analysed results of firmness in perch RF and PF are shown in (Figure 3). In PF we found a significant decrease ($p<0.05$) of resistance on the 8th day. On the other hand, RF there was no statistical difference during the whole storage time. In addition, from time to time the comparison between RF and PF firmness showed no significant differences except on the 8th day ($p<0.05$) but generally, RF had a higher firmness compared to the PF.

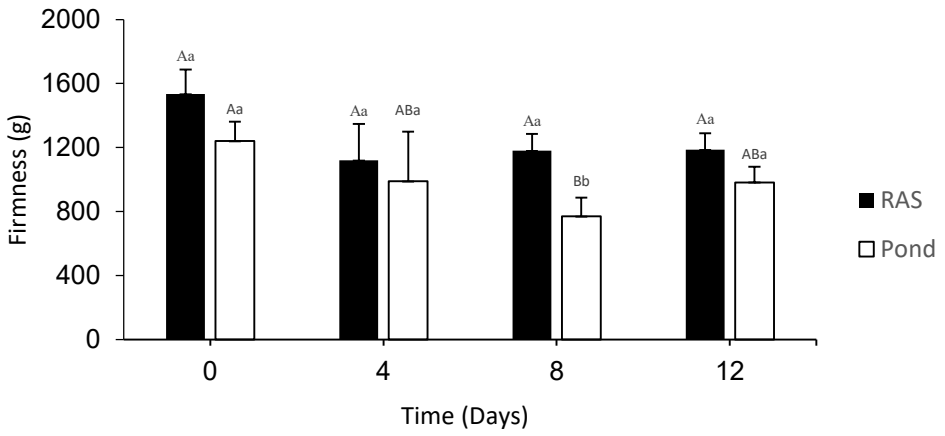

Figure 3. *Firmness of fish fillets from RAS and pond during refrigerated storage at +3.5°C (mean ± S.D., n=6). Data on firmness is expressed as gram (g). Different capital superscript letters above a bar indicate significant differences (p<0.05) in each rearing system. Small superscript letters above a bar indicate significant difference (p<0.05) between rearing systems at the same time point.*

3.8. Drip loss

Variations in drip loss changed significantly ($p<0.05$) during 132 hours storage at +4°C from (0.88 to 5.29% and 0.69 to 4.15%), in the RF and PF respectively (Figure 4). Also, comparing time to time between RF and PF did not show any significant difference ($p<0.05$) but we observed positive regression between drip loss and time in the RF and PF ($R=0.99$ and $R=0.98$) respectively.

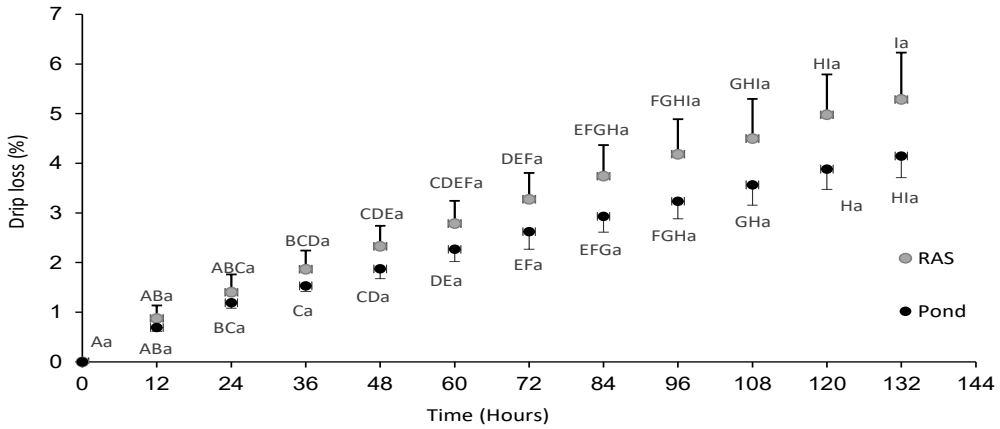

Figure 4. *Changes in drip loss of fish fillets from RAS and pond during 140 hours refrigerated storage at +3.5°C (mean ± S.D., n=6). Different capital superscript letters above a bar indicate significant differences ($p<0.05$) in each rearing system. Small superscript letters above a bar indicate significant difference ($p<0.05$) between rearing systems at the same time point.*

3.9. | Principal component analysis plot (PCA)

The PCA correlation loadings plot was used to visualize the correlation between the chosen measured parameters (oxidation, firmness, pH, rigor mortis, colour and drip loss parameters) (Figure 5a). PC 1 explained 63% of the variation, whereas PC 2 explained 25%. A clear correlation of the carbonyls, rigor mortis and firmness were demonstrated.

Firmness, however, was negatively correlated to the carbonyls, and both are explained mainly by PC 2, with correlation loadings of -0.45 and 0.35, respectively (Figure 5a), showing that higher protein oxidation has a negative affect on firmness. TBARS was less correlated to the mentioned factors.

Additionally, figure (5b) confirmed the results our study. In case of TBARS and drip loss we could not observe any difference by the effect of rearing systems. On the other hand, pH, carbonyls, rigor mortis, colour and firmness showed significant differences with respect to the rearing systems.

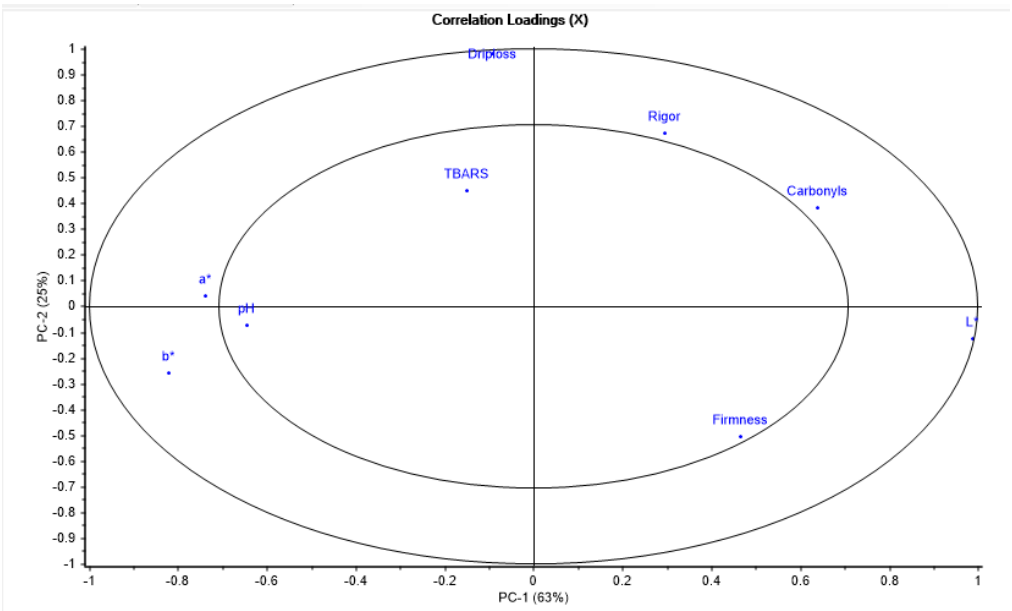

Figure 5a. *PCA plot showing the correlation loadings on PC 1 and PC 2 of the oxidation parameters MDA and carbonyls, pH, drip loss, rigor mortis, colour and firmness parameters of perch fillets.*

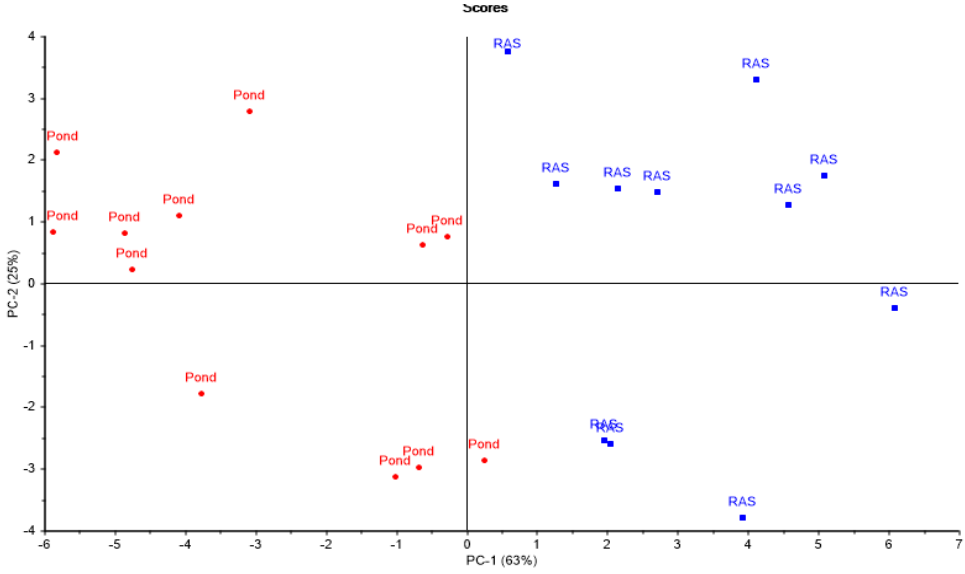

Figure 5b. *PCA plot showing the correlation loadings on PC 1 and PC 2 of the oxidation parameters MDA and carbonyls, pH, drip loss, rigor mortis, colour and firmness parameters of perch fillets between two different rearing systems.*

4. Discussion

In agreement to our pH results, Love (1976) reported the value between 6.7 to 7. In contradiction with our results, (Ocano-Higuera et al., 2009), reported lower pH in the initial day (6.43). This variations is most probably due to difference in species, diet, season and level of activity. Results for both rearing systems showed a trend of increasing pH during the storage time. This might be related to bacterial activities which lead to the production and accumulation of ammonia in fish muscle. According to the obtained results, the perch fish fillet from both rearing systems kept the satisfactory quality during 6 days storage at +3.5ºC. With respect to the temperature, the lower pH in the RF compared to the PF is mostly related to the higher metabolism and quicker depletion of cells stored energy, followed by a quicker onset of rigor mortis. Grigorakis, Alexis, Taylor, & Hole (2002) reported that the energy consumption is higher in farmed fish compared to wild fish. High post mortem pH in the fish fillet (generally greater than 6.0) is due to that fish fillet has a minimum percent of carbohydrates (lower than 0.5%) and therefore only small amounts of lactic acid can be produced after death (Gram & Huss, 1996).

On the other hand, comparing the results of pH between RF and PF showed a similar trend, but PF had significantly higher pH after 72h storage rather than RF. The differences can be explained with the difference between the diet and microbial activities during the storage time. Due to microbial activities the amount of ammonia increased in the PF compared to RF which leads to enhance the pH.

Onset of rigor mortis in the RF was faster than in PF. Furthermore, RF in this study reached full rigor mortis after 12 h earlier than PF.

The rigidity of rigor mortis is caused by cross-bridge between actin and myosin (Currie & Wolfe, 1979) and as a lack of adenosine triphosphate (ATP) which is needed to transport Ca^{2+} those bindings are not regenerated. In fillets from both rearing systems rigor mortis started fast due to the small fish size. Generally, small fish goes to rigor faster than bigger fish. On the other hand, the differences between the onset of rigor mortis in the RF and PF can be related to the different age and accumulated water temperature. PF were bigger than RF. This might be linked to a higher energy content and delay in the onset of rigor. In addition, RF was reared in the higher water temperature which leads to accelerate the onset of rigor. Abe & Okuma (1991), reported that carp from the cold waters showed a delay in the onset of rigor mortis compared to those from warmer water. However, according to the present results, it seems that rearing systems did not influence on the onset of post mortem changes.

Percentage of fat content were similar in the fillets from both rearing systems. Probably, different diet could not make a major impact on the fat content in the fish.

The fatty acid profiles results showed significant differences between both systems. In agreement with our results Jankowska, Zakes, Zmijewski, & Szczepkowski (2010) did not find any significant differences between the SFA proportion of reared and wild perch. On the other hand, Oku et al. (2009); Stejskal et al. (2011) reported that the amount of SFA in the Japanese eel (*Anguilla japonica*) and Eurasian perch (*Perca fluviatilis* L.) respectively were higher in the PF compared to RF.

The similar patterns to our study with myristic acid (C14:0) and stearic acid (C18:0) contents have been reported earlier by (Jankowska et al., 2010; Mairesse et al., 2007). Palmitic acid (C16:0) was the same in the RF and PF but showed the higher proportion compared to the mentioned SFA in both systems. Palmitic acid has been recognised as the predominant SFA in other fish species as well (Mairesse et al., 2007; Oku et al., 2009).

Additionally, the differences between MUFA in both systems were significantly, and RF showed a higher proportion. Jankowska, Zakes, Zmijewski, & Szczepkowski (2003); Jankowska et al. (2010); Stejskal et al. (2011) reported significantly higher proportion of MUFA in the fillet from cultivated pike perch and perch compared to PF.

In both groups oleic acid (C18:1) was identified as the prominent MUFA. In the line with our results Jankowska et al. (2010) and Stejskal et al. (2011) have shown significant higher amount of oleic acid in the fillet from cultivated and RAS compare to the PF. The difference might be related to the difference feed contents.

The total content of PUFA were higher in the PF compared to RF in the present study, in contradiction with (Jankowska et al., 2003; Stejskal et al., 2011). In agreement with our results Jankowska et al. (2010) reported higher levels of PUFA in wild perch compare to the cultivated perch. Docosahexaenoic (DHA; C:22: 6n-3) and eicosapentaenoic (EPA; C:20:5n-3) identified as predominant n-3 polyunsaturated acids in both systems. In addition, linoleic acid (18:2n-6) recognised as a major n-6 polyunsaturated in both systems.

As the total amount of PUFAn-3 and PUFAn-6 acids, did not show significant difference in the RF and PF the Σn-3: Σn-6 ratio were not differ in both systems. Sağglık et al. (2003) showed that the amount of arachidonic acid (C20:4n-6) was the higher in the tissue of pond sea bass while, linoleic acid (C18:2n-6) was the higher in cultivated fish and the n-3/n-6 proportion was higher in the pond fish.

The result of fatty acids in both groups showed higher amount of C18:1 n-9, C18:2 n-6 and Σn-3 in the RF rather than PF which confirm the highest content of vegetable oil supplementation in the RAS feed. Regarding to the essential fatty acids, RF showed higher amount of linoleic acid and PF illustrated higher content of alfa linolenic acid. It seems that both fillet characterised by the same nutritional value.

Protease enzymes are responsible to enhance the level of free amino acids in fish and other sea foods especially with increasing the storage time (Makarioslaham & Lee, 1993).The content of polyamines such as SPD and SPM decrease together with the deteriorating higher temperature (+12°C) along with prolonged storage time. In line with our results Krizek, Vacha, Vejsada, & Pelikanova (2011) reported the same trend in the amount of BAs in three different fish species (rainbow trout, Common carp (*Cyprinus carpio*) and perch (*Perca fluviatilis*) at two temperatures (+3 and 15°C). Aflaki, Ghoulipour, Saemian, Shiebani, & Salahinejad (2017) found an increasing trend for three different fish species at +4°C. The availability of decarboxylase enzymes, free amino acids and microbial growth can be considered as important factors on the amount of BAs (ten Brink, Damink, Joosten, & Huis int Veld, 1990).

Except for histamine and tyramine, currently there is no suggestions about the critical levels of (PUT), (CAD), (SPD) and (SPM) have been recommended for human consumption (Prester, 2011). According to the US Food and Drug Administration (FDA) the acceptable amount of histamine in fish flesh is 50 mg kg-1 ((USFDA), 1996) and the samples which were stored at +3.5°C from both systems did not reach this critical amount. On the other hand, storage at +12°C reached the critical level of histamine after 7 days in the fillet from pond system and 14 days in the fillets from RAS. 100–800 mg kg^{-1} of tyramine consumption is acceptable for adults, and more than 1080 mg kg^{-1} is considered as toxic (Tenbrink, Damink, Joosten, & Tveld, 1990).

In the present study we found increasing values for both lipid and protein oxidation in RF and PF during refrigerated storage for 12 days. The oxidation values (TBARS and carbonyl content) were higher in the RF, indicating that the development and progress of oxidation was faster compared to the PF.

In agreement with our results Chaijan, Benjakul, Visessanguan, & Faustman (2006) reported an increasing trend for MDA during 15 days of storage on ice in sardine fillet (*Sardinella gibbosa*). In opposite (Bahmani et al., 2011) could not observe a significant increase in the amount of TBRAS for whole ungutted golden gray mullet (*Liza aurata*) during 16 days in two different storage conditions (refrigerator and ice storage). In our study RF generally had higher MDA values but not significantly. However in both groups, lipid oxidation was minimal and below the proposed acceptable limits for human consumption (1.5 mg MDA kg^{-1}) (Ke, Cervantes, & Roblesmartinez, 1984). It seems that, both fillets had the same stability against lipid oxidation during the storage time.

In agreement with our results on protein oxidation, previous investigations on ten species of Mediterranean fish during 7 days of ice storage showed an increment in carbonyls group for all fish species (Passi, Cataudella, Tiano, & Littarru, 2005). Furthermore, over time the carbonyl results between RF and PF showed significant differences, indicating the progress of protein oxidation in RF was higher than PF. This might be due to higher antioxidants consumption in the fish reared in pond system.

The lack of ATP in the period of post mortem in fish fillet and anaerobic conditions are led to the antioxidants consumption which are important for the oxidation development. Probably, during post-mortem time, protein metal catalysed oxidation is dominated first, follow with the development of lipid oxidation. We would say that in our study, maybe protein oxidation was started earlier rather than lipid oxidation or both were in parallel.

In agreement with our colour results in PF, Ocaño-Higuera et al. (2011) reported a similar trend for L* parameter in ray fish during 18 days storage in ice. Furthermore Martelli et al. (2013) reported that, L* and b* parameters were higher in meagre fish which were reared in the tank system compared to the cage. In opposite, Jankowska et al. (2003) found, similar brightness and redness in the fresh fillet of pikeperch for both cultivated and from wild, while the yellowness in fish from aquaculture was higher than in wild fish.

In our study PF showed to be more reddish and darker, while RF were more white and light. This difference was even visually distinguishable. In line with that PF had higher a* parameter than RF indicating more redness in the fillet. This could be due to that, perch reared in pond can feed on zooplankton and zoobenthos which can increase the redness of fish fillet (Winfield, 2001). In addition, Mairesse, Thomas, Gardeur, & Brun-Bellut (2005) reported that fish reared in clear water's as in RAS, have a brighter colour. Furthermore, some authors have reported the interactions between fat content and L* value (Einen & Skrede, 1998; Morkore et al., 2001) which means that higher fat content coincides with lighter colour. Another possibility is due to, the less presence of a pronounced vascular system in perch fillet (Cox, 1998).

On the other hand, the C* value increased significantly and the b* value tended to increase during the refrigerated storage in RF but, in PF b* parameters are often associated with the development of lipid oxidation. Secondary products of lipid oxidation (aldehydes) can modify the stability of myoglobin and generate adducts through a covalent modification with myoglobin (Li & King, 1996). However, most probably, the higher yellowness in the fillets from the pond system compared to the RF is related to the presence of carotenoid in the diet of fish from the PF. The H*value was significantly higher in the RF compared to the PF.

Beside increased oxidation we found a decreasing hardness in the PF and increasing liquid loss in both fillets.

We observed a significant decrease in firmness in the PF after 8 days, which is in line with results by Wu, Zhu, Liang, Wang, & Li (2014) , who found decreasing hardness in red sea bream (*Pagrosomus major*) during 21 days storage at 0°C. Martelli et al. (2013) investigated the effects of two rearing systems (offshore sea cage vs land-based tank filled with geothermal water) on the meagre (*Argyrosomus regius*) fillet and found higher firmness in the muscle of fish reared in a tank. In opposite to our results Vácha et al. (2013); Stejskal et al. (2011)

reported that the textural profile in fresh fillet of perch which reared in the pond was higher than fresh fillet of perch reared in RAS. The decreasing firmness over storage time could be caused by the increased oxidation and subsequent denaturation of proteins. Torres-Arreola, Soto-Valdez, Peralta, Cardenas-Lopez, & Ezquerra-Brauer (2007) demonstrated that, protein oxidation leads to alterations in fish fillet texture and WHC, which can affect the integrity of the fillet fibers. Furthermore, in the last time point of storage (day 12), we observed an increasing trend in the fillet firmness form both rearing systems. This might be related to the proteolytic enzyme activity. Additionally, amplification of the myofibrillar structure through the formation of MP cross-linking can be considered as another factor (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010).

In addition, firmness in RF was higher than in PF. However this difference was only significant on day 8. One possible reason could be related to the higher crosslinked proteins in the RF rather than PF. Furthermore, the difference in rearing water temperature in the different systems can be considered. Water temperature is an important factor which can have an influence on muscle morphology by affecting the size and number of muscle fibers; at higher temperature of water the fibers will be thinner and their density will be increased (Hallier, Chevallier, Serot, & Prost, 2007). With a higher fiber density hardness increases (Hatae, Yoshimatsu, & Matsumoto, 1990). Hence, the higher water temperature in RAS compared to the ponds might play a role in the observed higher hardness in RF. Another reason that could explain the firmness differences between RF and PF is based on the fact that the collagen content increases with age and higher collagen content results in lower hardness (Fauconneau & Laroche, 1996). As the fish from the pond were age 2 + while the faster growing fish from RAS were age 1+ the lower firmness in fillets from pond fish could also be due to a higher collagen content.

The results of PCA indicated that the firmness, is negatively correlated to the carbonyls, confirming our conclusion that higher protein oxidation (higher amount of carbonyls) leads to changing secondary and tertiary structure and decrease firmness. On the other hand, rigor mortis and drip loss are correlated to carbonyls due to the cross linked protein during most mortem time. Furthermore, pH is negatively correlated to other parameters, indicating that it does not have a significant impact on firmness, drip loss, rigor mortis and oxidation. Regarding the relationship of lipid oxidation to colour, the low correlation in PC 1 and PC 2 is in line with the conclusion that the values were low.

According to our results we found a positive correlation between drip loss and time in RF and PF (r=0.99 and r=0.98 respectively). In agreement with our results, Martelli et al. (2013) reported a higher drip loss in the muscle of meagre fish reared in a tank system compared to the muscle of meagre fish reared in a cage system. It has been reported that the liquid-holding capacity declined during the storage time throughout chilled storage in various fish species including farmed and wild rainbow trout and gilthead sea bream (Morkore, Hansen, Unander, & Einen, 2002). This can also be related to the higher firmness in RF as Rawdkuen, Jongjareonrak, Phatcharat, & Benjakul (2010) reported a correlation between firmness and liquid loss.

In general, protein oxidation has a decreasing effect on the water holding capacity (WHC). Because the ability of the myofibrillar proteins to keep water in muscle increasing oxidation of proteins will result in increased free water and decreased WHC (Sikorski & Olley 1976). Furthermore, in our study we observed a high correlation of increased drip loss and protein oxidation in the RF and PF (r=0.91 and r=0.92 respectively). Probably, the development of protein degradation had a high impact on the muscle structure and increased drip loss. Due to protein degradation sulfhydryl (SH) group are converted to the disulfides bonds which have negative impact on firmness and WHC parameters (Huff-Lonergan & Lonergan, 2005).

5. Conclusions

This study elucidated the importance of the fish rearing conditions on the development of protein and lipid oxidation and some other quality aspects in fish fillets stored at +3.5°C. Lipid and protein oxidation products were higher in the fillets from RAS and increased during the refrigerated storage. During post-mortem changes protein oxidation dominated sooner than lipid oxidation. BAs showed an acceptable microbial quality in the fillets from both systems which were stored at +3.5°C. Increasing drip loss over time indicates the effect of protein denaturation on muscle integrity in the present study, while decreasing firmness illustrates protein denaturation. Higher firmness and liquid loss in the fillets from RAS might be related to the cross-linked proteins. However, rigor index showed a faster onset of rigor mortis in the fillet from RAS compared to the pond, which could be linked to the higher cell energy. Finally, it seems that pond fillets showed better textural quality compared to the RAS fillets due to higher antioxidants consumption.

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CHAPTER 6

GENERAL DISCUSSION

High proportion of PUFA in fish muscle makes it prone to lipid peroxidation. Beside the PUFA, which are prone to oxidation by reactive oxygen species (ROS), some amino acids, such as cysteine and methionine, are also the first targets of ROS attack (Lii, Chai, Zhao, Thomas, & Hendrich, 1994; Vogt, 1995). Therefore, ROS can be considered as an initiating factor for both lipid and protein oxidation (Xiong, 2000). Secondary products of lipid oxidation are mostly linked to the unpleasant off-flavours and odour, while development of protein oxidation leads to alteration in the secondary and tertiary protein structures followed by textural changes (Wasowicz et al., 2004; Zhang, Xiao, & Ahn, 2013). Fish muscle quality parameters, such as taste, colour and texture, are evaluated with respect to the lipid and protein oxidation progress in the present thesis.

The assessment of pre-/in-/post- rigor mortis alterations were performed to determine a correlation between the processing of fish after death and shelf life of the products. For this purpose, storage at low temperatures has been used as a common method to retain the fish muscle quality. The optimum time of storage depends on the fish species (fatty or lean fish) and can be from a few days in the refrigerator to a few months in the freezer (Bahmani et al., 2011; Hematyar, Masilko, Mraz, & Sampels, 2018). Lower storage temperatures compared to higher temperatures usually guarantee a better fish muscle quality during the storage time (Passi, Cataudella, Tiano, & Littarru, 2005; Gandotra, 2012). Concerning our studies on fish muscle quality, we considered some quality parameters such as firmness, WHC as well as lipid-protein oxidation progress as signs of freshness. Regardless of storage conditions and fish species, we observed that the amount of MDA and carbonyls as indicators of lipid and protein oxidation respectively increased significantly, while firmness and WHC decreased considerably in both common carp and perch muscle during the storage time. The same trend had been reported before on different fish species (Baron, Kjaersgard, Jessen, & Jacobsen, 2007; Cheng, Sun, & Zhu, 2017; Lu, Wang, & Luo, 2017; Passi, et al., 2005; Yin, Luo, Fan, Wu, & Feng, 2014).

The precise mechanisms and pathways of protein oxidation as well as lipid-protein interactions during storage time of fish muscle are still unclear. Additionally, there is a doubt if lipid and protein oxidation are started together, or if one of them is started earlier. Several factors can be marked as initiator factors to onset lipid and protein oxidation (Bernardini et al., 2011). Depending on the storage conditions, one or more initiators are responsible for starting the oxidation progress; however, the primary and secondary targets of oxidation can vary (Hematyar, et al., 2019). Some authors suggested that increasing the ROS level might play a key role for fish muscle deterioration (Bekhit, Hopkins, Fahri, & Ponnampalam, 2013; Li & Liu, 2012). For instance, the most reactive ROS is hydroxyl radical ($HO^{\cdot}$), which is generated via the Fenton reaction route. After stunning the fish, activity of protease enzymes and the pH decrease are suggested to inhibit the cellular controlling mechanism for the metal ions formation (Gupta & Chaudhury, 2007). Therefore, during the storage time $HO^{\cdot}$ has high contribution and leads to the deterioration of muscle quality through oxidation processes (Bekhit, et al., 2013). Subsequently, the amount of lipid and protein oxidation products are considered as indicators of oxidation progress during food storage (Baron, Kjaersgard, Jessen, & Jacobsen, 2007; Estévez & Cava, 2004). However, good slaughter conditions (bleeding), treating the fish muscle with antioxidants, and adequate storage can prevent or postpone the oxidation development (Afonso, Bandarra, Nunes, & Cardoso, 2016; Heinonen, Meyer, & Frankel, 1998; Hematyar, et al., 2018; Maqsood & Benjakul, 2011).

Thus, we examined and reported the oxidation progress in common carp and perch muscle during frozen and refrigerated storages. Our results showed a significant increase in the amount of TBARS and carbonyls in the muscle of both examined fish species. However, TBARS

did not reached to the critical amount (1.5 mg MDA kg^{-1}) (Ke, et al., 1984). Leygonie, Britz, & Hoffman (2012); Thanonkaew, Benjakul, Visessanguan, & Decker (2006) reported the same trend for lipid and protein oxidation in fatty and lean fish. Although carp is a moderate fatty fish (8%) and perch is a lean fish (1%), but most probably due to the less amount of PUFA (12%) in the carp muscle compared to the perch (RAS and pond systems) muscles (43% and 47%) respectively, we observed fewer lipid oxidation products (MDA) in carp muscle compared to perch muscle. Furthermore, higher lipid and protein oxidation products in the muscle from RAS compared to the muscle from pond fish might be related to the higher consumption of natural antioxidants in the pond fish diet. Additionally, in several published papers a good relationship between lipid and protein oxidation products in fish (Soyer & Hultin, 2000) beef (Estévez & Cava, 2004) and chicken (Soyer, Özalp, Dalmış, & Bilgin, 2010) has been reported. Moreover, we could not observe a good correlation between TBARS and carbonyls in the carp muscle, while in the perch muscles (RAS and pond) we reported a positive and significant correlation. Probably, the progress of lipid and protein oxidation in carp muscle was independent and not influenced by each other. On the other hand, this trend was simultaneous and connected in the perch muscles from both rearing systems. It seems that the formed products of oxidized lipids and proteins can then further boost oxidation of both types (TBARS and carbonyls). In addition, with respect to the storage conditions (-20°C and +4°C) in the perch experiments, we observed the same trend for lipid and protein oxidation development. However, in both storage conditions (-20°C and +4°C) we had a better correlation between TBARS and carbonyls in the fillets from RAS fish (0.98 and 0.96) compared to fillets from pond fish (0.97 and 0.94) respectively. We conclude that probably due to the higher interaction between lipid and protein oxidation products we had a higher amount of oxidised products in the muscle from RAS fish compared to the muscle from pond fish. Comparisons between RAS and pond muscles revealed significant differences in the amount of carbonyls at time 0 and 4 months during frozen storage, while we observed a significant increasing trend during the whole refrigerated storage, indicating a more intense protein degradation in the muscle from RAS compared to the muscle from a pond. However, during the frozen storage, protein denaturation started faster in the muscle from RAS compared to the muscle from pond fish until 4 months of storage. Additionally, we observed a significant difference of carbonyls in perch muscles after 8 days, while a marked increase in TBARS was detected after 12 days of storage at +4°C in both rearing systems. Thus, probably protein degradation started earlier, followed by lipid oxidation. On the other hand, a marked increase for both lipid and protein oxidation products in the muscles from RAS and a pond was detected after 4 months of frozen storage. Most likely, during frozen storage lipid and protein oxidation started together.

Colour is directly linked to the oxidation development and affects consumer perception. For this purpose, we measured the colour in carp and perch muscles with respect to the L*, b* and a* parameters. We observed an increasing trend in L*, constant increase in b*, and also a fluctuated increase in a* factors during the storage of carp muscle. Both L* and b* are mostly connected with the progress of lipid oxidation. Schubring (2005) reported a significant increase in both L*and b* factors in cod (*Gadus morhua*) muscles during 13 months storage at -20°C, while a wave-like pathway for a* value was found. As mentioned above, a lower lipid oxidation progress in the carp muscle might be a reason for constant increase of b*. On the other hand, fillets from the perch in RAS fish showed higher L* and b*, whereas fillets from perch from pond fish had a distinguishably higher a* value. Martelli, et al. (2013) reported that L* and b* factors were higher in the muscles of meagre (*Argyrosomus regius*) which were reared in a tank in comparison to the muscles from fish from a cage system. Most probably, this was due to the different available feed sources. Fish reared in pond system can feed on zooplankton and zoobenthos that can enhance the redness of fish fillet (Winfield, 2001).

Additionally, Mairesse, Thomas, Gardeur, & Brun-Bellut (2005) revealed that fish reared in clear waters like in RAS showed a brighter muscle colour.

Furthermore, we investigated firmness and liquid loss of the two different fish species with respect to elapsed time. Our results in both carp and perch muscle showed a decreasing trend for firmness and WHC over the storage time. The results are in line with Lu, et al. (2017); Wu, Zhu, Liang, Wang & Li (2014). Additionally, probably due to more cross-linked proteins, muscle from RAS revealed higher firmness and liquid loss rather than the pond one. In agreement with our results, Martelli et al. (2013) reported a lower liquid loss in fillet from meagre reared in a cage system compared to the fillets from meagre from a tank system. According to our results, we found a significant positive correlation between liquid loss and carbonyls as indicators of protein oxidation during frozen (r=0.89 and r=0.90) and refrigerated (r=0.91 and r=0.92) storages in the muscles from RAS and the pond systems respectively. In opposite we could not find a good correlation between WHC and protein oxidation in the carp muscle (r=0.64). We concluded that probably in the perch muscle textural parameters were influenced by protein oxidation, whereas in carp muscle cell disruption as a result of formation of ice crystals followed by protein denaturation had a higher impact. Additionally, we found a negative correlation between firmness and carbonyls in both fish species. This means that increased protein oxidation is linked to the reduction of firmness. However, we could not observe a good correlation between firmness and carbonyls during refrigerated storage. This result indicates that, most probably firmness is connected to the protein degradation influenced by calpain enzymes activities (Koohmaraie, 1996). Also, increased firmness on the last day of refrigerated storage might be related to the protein degradation followed by protein aggregation in the muscles from both rearing systems. Additionally, higher firmness in the perch muscle stored at +4°C compared to those stored at -20°C even after 10 months confirmed more protein aggregation as well as more cross-linked proteins during post mortem time rather than the frozen time. We assumed that, moderate protein oxidation in fish muscle can reduce the firmness owing to the more unfold protein structures, while extensive oxidation often leads to higher firmness because of more aggregated proteins. Myofibril proteins play key roles on fish muscle quality (Lonergan, Topel, & Marple, 2019). Therefore, the formation of cross-link proteins, beside the effect of lipids-proteins oxidation, is responsible for changing the fish muscle quality. Further, formation of unfolded proteins during frozen storage due to oxidation or lipid-protein interaction can be considered as responsible factors for the WHC and firmness reduction. Additionally, muscle from a pond showed a better stability until 4 months storage at -20°C compared to the RAS one. Calpain activity decreased in the fillets from both rearing systems during the storage time. In agreement, Nagaraj & Santhanam (2006) reported the same calpain activity trend in the goat muscles during 120 days frozen (-15°C) storage. The enzyme results confirm that protein denaturation in our experiment is mostly connected to the ice crystal formation in fish muscle during frozen storage rather than other mechanisms. Additionally, the fillet from pond showed a higher calpain activity but not significantly compared to the fillet from RAS, which is in agreement with our firmness results. Probably, higher cross-linked proteins and oxidation progress might be the reason for a lower calpain activity (Lametsch, Lonergan, & Huff-Lonergan, 2008) in the fillet from RAS compared to the fillet from a pond system.

Proteomic analyses have been performed on the perch muscle during 10 months storage at -20°C. SDS-PAGE analysis showed protein denaturation in the muscle from both rearing systems (RAS and pond) during storage time. In line with that the intensity of actin and myosin bands in the RAS and pond muscles were significantly decreased. Corresponding to our results, Jasra, Jasra, & Talesara (2001) reported MLC and α-actin denaturation in carp (*Labeo rohita*) muscle during 6 months storage at -20°C. It is known that in the muscle foods, actin

and myosin are very susceptible proteins to oxidation (Donagh, Tyther, & Sheehan, 2005). An investigation on cod muscle showed that 80% of myosin is deteriorated during frozen storage (Sharp & Offer 1992). Our SDS-PAGE results confirmed that protein denaturation occurred in the mentioned proteins particularly, after 8 and 10 months storage. Additionally, we found out that some other proteins, such as nebulin and troponin, were decreased after 8 and 10 months, which can affect textural parameters. Further, our native page results showed lower intensity in the proteins of muscle from pond fish compared to muscle from RAS, especially after 8 and 10 months storage. We observed fainted protein bands in native page between 480-720 kDa, which is linked to the myosin denaturation, in the muscle from pond fish, while in the muscle from RAS fish it was stable. The native page results showed more stability and sulphide bonds in the muscle proteins of RAS fish compared to muscle from a pond. Regarding our immunoblot results, we observed that proteins with a size between 40 to 43 kDa were oxidized heavily. In agreement with our results, Baron, et al. (2007) reported oxidized protein carbonyl groups at 35 and 200 kDa in the rainbow trout (*Oncorhynchus mykiss*) muscle over the 13 months storage at -20°C. Our western blot results confirmed the vulnerability of actin and myosin proteins during frozen storage, especially after 8 months storage. Proteolytic degradation specially, calpain activities (Tseng et al., 2003), beside the influence of ice crystal formation on cell disruption, are the main factors of fainted bands during storage time (Subbaiah, 2015). Considering the proteomics results on the perch muscle from both rearing systems showed more resistance against protein denaturation in the muscle from pond fish, particularly up till 4 months storage compared to muscle from RAS fish. Less bond intensity and fewer oxidised carbonyls in the muscle from a pond system compared to those from RAS means that maybe fewer proteins were oxidised but they were oxidized more heavily. Dalsgaard, Otzen, Nielsen, & Larsen (2007) reported that, random coiled proteins in comparison to globular proteins are more susceptible to oxidation. In line with our result, Eymard, et al. (2009) reported that due to the different structures probably, globular proteins are protected more against oxidation in the period of storage time. We conclude that due to the higher presence of natural antioxidants and also probably a more tight and well-defined protein structure, muscles from pond fish are more protected against oxidation compared to muscles from RAS fish. Also, we found higher cross-linked proteins in the muscle from RAS fish compared to the muscle from pond fish, which confirm higher liquid loss and firmness in the muscle from RAS fish. Moczkowska et al. (2017) also reported higher firmness is linked to the higher cross-linked protein in beef. Furthermore, a major reduction in firmness occurred after 4 months storage in RAS and pond fillets (43 and 40%) respectively however, in the reminding times it was not marked. Immunoblot result showed less oxidised carbonyl until 4 months compared to 8 and 10 months storage at -20°C while higher firmness reduction observed in the first 4 months of storage. It seems that, ice crystal formation can be considered as a main role compared to protein denaturation in the first months of frozen storage but in the last months of storage time protein denaturation is dominated. Concerning the final quality of the fillets from both rearing systems after 10 months storage at -20°C, we observed a better texture and fewer lipid- and protein oxidation products in the fillets from pond fish.

In summary, our results indicated that the development of lipid and protein oxidation was simultaneously progressing during frozen storage. During short term storage first protein oxidation dominates and is followed by lipid oxidation. Furthermore, the amount of PUFA should be considered as an important factor rather than the amount of fat content in fish muscle. It seems that higher PUFA makes fillets more prone to oxidation and results in a higher interaction between lipid-protein oxidation products. Moreover, during post-mortem time textural parameters are mostly connected to the autolytic enzyme reactions followed by

protein degradation, while during frozen storage texture is linked to the protein denaturation. This study showed that the current available methods to investigate lipid and protein oxidation as well as their interactions are not sufficient. It seems that more sensitive methods are needed to investigate protein oxidation more deeply. Additionally, supplementary feeding such as cereals and vegetable oils in carp is a way to produce omega carp and increase the amount of PUFA. However this may lead fillet more prone to oxidation during the storage time. It has been shown before Stéphan et al. (1995) that increasing the amount of α-tocopherol decreased the progress of lipid oxidation during frozen storage. Therefore, optimizing the amount of antioxidants in fish fillet and the comparison between the effect of different diets on fish fillet can be consider for future works. Furthermore, lipidomics methods such as mass spectrometry can provide a comprehensive vision of lipids in nutrition. Lipidomics is used to find the effect of diet on the composition, function and structure of lipids. Lipidomics as an advance method is suggested to use for more investigations on bioactive mediators which are produced from dietary with ω-3 PUFA in fish fillet.

CONCLUSIONS

1) According to our studies, fish species with a high amount of PUFA were very prone to oxidation; therefore, long term storage under frozen condition is not suggested.
2) We showed high stability of common carp muscle during long term storage at −20°C most probably due to the low amount of PUFA in the muscle. Also, a higher amount of natural antioxidants in the feed of fish which were reared in pond system might be a reason for fewer lipid and protein oxidation products in their muscle rather than muscle from RAS.
3) We propose that the products of lipid oxidation promote protein oxidation in fish rather than the other way around specially, during frozen storage, while during post-mortem changes protein oxidation dominates.
4) The proteomic approach indicated that actin and myosin are very prone to oxidation in fish muscle followed by nebulin and troponin, which are responsible for WHC decline and firmness.
5) Probably, during first months of frozen storage formation of ice crystal can be considered as the main factor which has negative effect on textural parameters then, protein denaturation is dominated.
6) We reported higher cross-linked proteins in the fillet from RAS fish rather than the fillet from a pond fish system, which indicates higher liquid loss and firmness in the fillet from RAS fish.
7) We suggest that fillet from pond fish contained higher natural antioxidants and probably, tighter and well-defined protein structure.
8) Optimizing the amount of antioxidants in the artificial fish diet should be considered in further studies in order to avoid the oxidation progress in fish fillets.

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ENGLISH SUMMARY

Protein and lipid oxidation in fish: pathways, kinetics and products

Nima Hematyar

Fish muscle contains omega-3 fatty acids and high quality protein and other valuable nutrients. On the other hand, due to the high content of water, neutral pH, presence of PUFA and sensitive amino acids, fish muscle is a very perishable food. Therefore, storage conditions can be considered as an important parameter in order to keep the fish muscle quality. However, during storage, fish muscle can undergo unpleasant changes which may restrict the shelf life. The progress of lipid and protein oxidation as well as interaction between the oxidation products could have a negative role during storage time.

Negative effect of oxidation on the fish fillet quality has been reported by several authors, but still the pathways and products of protein oxidation owning to the diversity of the protein oxidation products is not clear. Pathways of lipid and protein interaction, target amino acids, storage conditions and also, final impact on the fish muscle quality have been investigated in this study.

To ensure that product quality is retained during production and storage, it is important to have insight into specific processes happening. We examined the quality of fish muscle in common carp and perch during different storage conditions (+4°C and -20°C). Also, we compared some quality parameters in perch muscle from two different rearing systems to identify the pathways and products of oxidation. According to the storage condition the important initiator for muscle fish deterioration as well as first target of oxidation can be vary. For this propose, we examined possible alterations in fish muscle during the storage time. The present study investigated the oxidation progress with respect to TBARS and carbonyls as markers of lipid and protein oxidation respectively as well as contribution of PUFA on the lipid oxidation development. Further, the effect of storage conditions and oxidation on the textural and sensorial parameters were examined. Possible changes of pH, rigor index and biogenic amines, during post mortem were studied. Additionally, we performed proteomic analysis in order to investigate more deeply the protein alteration and find a correlation between protein structures and fish muscle quality.

Our results showed a good stability in common carp (*Cyprinus carpio*) fillet during 6 months storage at -20°C. According to our results, we observed a significant decrease in the firmness of common carp muscle after 1 week and then it was constant while liquid loss increased significantly with storage time. Regarding the sensory parameters, we could not find any differences in the examined parameters in both stored raw and cooked fillets compared to the fresh one. Although, both lipid oxidation parameters (TBARS and PV) were increased significantly but it did not reach the critical level for human consumption. On the other hand, protein oxidation which is involved in WHC deterioration was increased significantly. In addition, we investigated the muscle quality of Eurasian perch (*Perca fluviatilis* L.) from two rearing systems (RAS and pond). During frozen and refrigerated storage, we observed a significant increase in the amount of TBARS and carbonyls in both rearing systems. Comparison between both rearing systems did not show any differences in the amount of TBARS while carbonyl was significantly higher in the RAS fillets rather than pond fillets particularly, until 4 month storage at -20°C. Also, we found a significant increase in liquid loss and considerable decrease in firmness in the fillets from both rearing systems. Our results showed higher firmness and liquid loss in the RAS fillets compared to the pond one. Proteomic analysis on the fillets from both rearing systems during frozen storage showed more stability in the RAS

fillet however, western blot revealed more oxidized carbonyls in the RAS fillets rather than pond fillet specially, at time 0 and 4. Probably, formation of ice crystals and accumulation of ROS have key roles on the development of oxidation during frozen storage while enzymatic activity follow by protein degradation are important during refrigerated storage. Additionally, digestibility of cross-linked protein in the human body might be interesting for the future research work.

CZECH SUMMARY

Oxidace proteinů a lipidů u ryb: dráhy, kinetika a produkty

Nima Hematyar

Rybí svalovina obsahuje omega-3 mastné kyseliny, vysoce kvalitní bílkoviny a další cenné živiny. Na druhé straně představuje rybí svalovina velmi rychle se kazící potravinu díky vysokému obsahu vody, neutrálnímu pH, přítomnosti polynenasycených mastných kyselin a citlivých aminokyselin. Proto mohou být podmínky skladování považovány za důležitý parametr nutný k zachování kvality svaloviny ryb. V průběhu skladování však může být svalovina ryb vystavena nepříjemným změnám, které mohou omezit její trvanlivost. Vyvíjející se oxidace lipidů a proteinů, jakož i interakce mezi produkty oxidace mohou představovat důležitý problém během skladování.

Informace o negativním účinku oxidace na kvalitu rybího filetu byly již zveřejněny několika autory, nicméně stále nejsou jasné dráhy a produkty oxidace proteinů kvůli různorodosti produktů oxidace proteinů. V této výzkumné práci byly zkoumány dráhy interakce lipidů a proteinů, cílové aminokyseliny, podmínky skladování a také konečný dopad na kvalitu svaloviny ryb.

Abychom zajistili uchování kvality produktu během výroby a skladování, je důležité porozumět konkrétním procesům, ke kterým dochází. Zkoumali jsme kvalitu rybí svaloviny u kapra obecného a okouna za různých skladovacích podmínek (+ 4°C a -20°C). Také jsme porovnávali některé kvalitativní parametry ve svalovině okouna ze dvou různých chovných systémů, abychom identifikovali dráhy a produkty oxidace. Podle podmínek skladování se může iniciátor zhoršení svaloviny ryb a primární cíl oxidace lišit. Z tohoto důvodu jsme zkoumali možné změny ve svalovině ryb v průběhu skladování. Tato práce zkoumala, jak se oxidace vyvíjí s ohledem na reaktivní látky kyseliny thiobarbiturové a karbonyly jako markerů oxidace lipidů a proteinů a jaký podíl mají polynenasycené mastné kyseliny na rozvoji oxidace lipidů. Dále byl zkoumán vliv podmínek skladování a oxidace na texturní a senzorické parametry. V průběhu postmortálních změn byly studovány možné změny pH, rigor index a biogenní aminy. Navíc jsme provedli proteomickou analýzu, abychom mohli hlouběji prozkoumat změnu proteinů a najít korelaci mezi strukturami proteinů a kvalitou svaloviny ryb.

Naše výsledky prokázaly dobrou stabilitu u filetu kapra obecného (*Cyprinus carpio*) během 6 měsíců skladování při teplotě -20°C. Podle našich výsledků jsme po jednom týdnu zaznamenali výrazný pokles tuhosti svaloviny u kapra obecného a poté byla konstantní, zatímco ztráta tekutiny se výrazně zvyšovala s dobou skladování. Co se týče senzorických parametrů, nenarazili jsme ve zkoumaných parametrech na žádné rozdíly u uchovávaných syrových či vařených filetů ve srovnání s čerstvými. Ačkoli byly oba parametry oxidace lipidů (TBARS a PV) významně zvýšené, nedosáhly kritické úrovně z hlediska lidské konzumace. Na druhé straně se zásadně zvýšila oxidace proteinů, která se podílí na zhoršení kapacity zadržování vody. Dále jsme zkoumali kvalitu svaloviny u okouna říčního (*Perca fluviatilis* L.) ze dvou chovných systémů (RAS a rybník). Během mraženého a chlazeného uchovávání jsme pozorovali významné zvýšení množství TBARS a karbonylů v obou chovných systémech. Srovnání mezi oběma chovnými systémy neprokázalo žádné rozdíly v množství TBARS, zatímco koncentrace karbonylů byla významně vyšší u filetů pocházejících z RAS než u filetů pocházejících z rybníka, zejména před uplynutím 4 měsíců skladování při -20°C. Zaznamenali jsme také značný nárůst ztráty tekutin a výrazné snížení pevnosti filetů z obou chovných systémů. Naše výsledky prokázaly vyšší tuhost a ztrátu tekutin u filetů pocházejících z RAS ve srovnání s filety pocházejícími z rybníka. Proteomická analýza filetů z obou chovných

systémů ukázala větší stabilitu u filetu pocházejícího z RAS během mraženého uchovávání, nicméně imunoblot odhalil více oxidovaných karbonylů u filetu pocházejícího z RAS než u filetů pocházejících z rybníka, obzvláště v čase 0 a 4. Tvorba ledových krystalů a akumulace ROS hraje pravděpodobně klíčovou roli ve vývoji oxidace během mraženého uchovávání, přičemž enzymová aktivita následovaná rozpadem proteinů je důležitá při chlazeném uchovávání. Stravitelnost propojených proteinů v lidském těle může navíc představovat zajímavý aspekt pro budoucí výzkum.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Hematyar, N., Rustad, T., Sampels, S., Dalsgaard, T. K., 2019. Relationship between lipid and protein oxidation in fish. Aquaculture Research 50: 1393–1403. (IF 2018 = 1.502)

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Abstracts and conference proceedings

Hematyar, N., Sampels, S., Dalsgaard, T.K., Mraz, J., 2018. Proteome changes of eurasian perch (*Perca fluviatilis*) fillet from two different rearing systems influenced by frozen storage. In: Book of abstracts "AQUA 2018", August 25–29, 2018, Montpellier, France, p. 344. (oral presentation)

Mohagheghi Samarin, Azadeh., Sampels, S., Policar, T., Rodina, M., **Hematyar, N.**, Mohagheghi Samarin, Azin, 2017. mRNA Expression changes during *in vitro* oocyte ageing in african catfish *Clarias gariepinus*. Aquaculture Europe 2017, October 17–20, 2017, Dubrovnik, Croatia. (poster presentation).

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Mohagheghi Samarin, Azadeh., Sampels, S., Policar, T., Rodina, M., **Hematyar, N.**, Mohagheghi Samarin, Azin, 2017. mRNA Expression changes during in vitro oocyte ageing in african catfish *Clarias gariepinus*. Aquaculture Europe 2017, October 17–20, 2017, Dubrovnik, Croatia. (poster presentation).	2017
Hematyar, N., Masilko, J., Mraz, J., Sampels, S., 2017. Effects of long term storage on nutritional value, oxidation and sensory parameters in fillets of common carp (*Cyprinus carpio* L.). In: Book of abstracts "15th Euro Fed Lipid", August 27-30, 2017, Uppsala, Sweden. (poster presentation)	2017

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