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Fatty acids as biomarkers of the influence of biological and environmental factors on marine organisms

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Index

1 Introduction

During my PhD I worked to implement scientific knowledge concerning the use of lipids as biomarkers, specifically I focused on fatty acids.

Lipids as biomarkers have already been used in several studies, for example to investigate: i) trophic interactions between different marine organisms (food chain) (Alfaro et al. 2006; Copeman et al. 2013; Dalsgaard et al. 2003; Mourente et al. 2015), ii) the biomass and the typology of population in marine sediments (Huang et al. 2015; Palomo and Canuel 2010), iii) the algal community of the sea ice (Fahl and Kattner 1993; Leu et al. 2010) and iv) the relationship between environmental pollution or stressful habitat conditions with the biological response of marine organisms (Ferreira et al. 2010; Fokina et al. 2013; Gonçalves et al. 2016).

During my research I have deepened the tropic aspects of marine organisms by using fatty acids (FAs) as biomarkers to study their response to climate change, since FAs are regarded as ideal biological markers of environmental exposure in marine researchs (Alfaro et al. 2006; Copeman et al. 2013; Dalsgaard et al. 2003; Könneke and Widdel 2003; Mourente et al. 2015; Sajjadi and Eghtesadi-Araghi 2011).

In the last years, the global warming has had a great impact on the scientific community, because of the effects that a temperature increase could have on the planet and on the living organisms (particularly on their physiology); Intergovernmental Panel on Climate Change (IPCC 2014) has foretold an increase between +3 and +6 °C of the surface of the earth. Particularly, in the Ross Sea region, Antarctica, shelf water warming up to 0.8-1.4 °C is predicted by 2200 (Timmermann and Hellmer 2013). This change is leading to macroscopic consequences, such as melting ice, but far more sneakily, it is leading to significant changes in organisms that have adapted to live in such extreme conditions.

The hypothesis of my research is that the thermal rise can influence the composition of fatty acids in different tissues of the Antarctic fish *Trematomus bernacchii*, a fish of the Nototheniidae family often used as a bioindicator in environmental studies. I also investigated if these changes are related to the occurrence of pathological conditions, or to the ability of the fish to become accustomed to a new environmental condition. To achieve this goal, we optimized and validated an analytical methodology modified from the literature, based on i) microwave-assisted extraction of lipids from a lyophilized sample,

ii) derivatization of lipid extract using $NaOCH_3$, and iii) FAMEs separation and identification by GC-MS.

This work is part of the project funded by the PNRA (Programma Nazionale di Ricerche in Antartide) entitled "Chemical Contamination and Assessment and evolution of the chemical contamination by organic and inorganic constituents in Antarctic coastal areas (project 2013/AZ2.01)"

In addition, during my PhD program, I had the opportunity to apply my knowledges on the determination of fatty acid composition in other projects:

- Study of the influence of size and sex of Atlantic Bluefin Tuna on the meet quality composition (project MIPAFF note 6775, Art.36 Paragraph 1 Reg (UE9 n 508/2014) to O.C).
- Variation of the gonadal lipid profile during the reproductive cycle of female crustacean *Penaeus kerathurus*.

2 Lipids

Lipids are one of the major four classes of organic compounds in biology systems along with protein, carbohydrates and nucleic acids.

Lipids serve as fuel molecules, as highly concentrated energy stores, as signal molecules, as components of cell membranes and carriers of fat-soluble vitamins, (e.g. A, D, E and K). Both lipids and lipid derivatives serve as vitamins and hormones.

Fats consist of numerous chemical compounds, including monoglycerides, diglycerides, triglycerides, phosphatides, cerebrosides, sterols, terpenes, fatty alcohols and fatty acids. Fatty acids are the main component of phospholipids, triglycerides, diglycerides, monoglycerides and sterol esters (Chow 2007). However other lipids, such as cholesterol and terpenes do not contain fatty acids, but these molecules are potentially related to fatty acids because they are synthesized from the catabolic end product of fatty acid degradation (i.e. acetyl CoA) (Puri 2014).

2.1 Lipids classification

2.1.1 Acylglycerols

The major reserve of lipids are triacylglycerols (formerly known as triglycerides).

Monoacylglycerols and diacylglycerols may also be present as minor components. However, these are important intermediates in the biosynthesis and catabolism of triacylglycerols and other classes of lipids (Gunstone 2012).

Monoacylglycerols (monoglycerides) are fatty acid mono esters of glycerol and they exist in two isomeric forms: α-monoglyceride (1 and 3-monoacyl-sn-glycerol) and β-monoglyceride (2-monoacyl-sn-glycerol). Pure isomers readily change to a 90:10 mixtures of the α- and β-isomers. This rearrangement is promoted by acid or alkali and is an example of transesterification (Gunstone 2012).

Diacylglycerols (diglycerides) are fatty acid diesters of glycerol and they also exist in two isomeric forms: αβ-diglyceride (1,2 and 2,3-diacyl-sn-glycerol) and αα'-diglyceride

(1,3-diacyl-sn-glycerol). They readily form an equilibrium mixture with 1,3-diacylglycerol being the more stable (Gunstone 2012).

Triacylglycerols are the simplest and the most abundant lipids, also referred to as fats, neutral fats or triglycerides. They are fatty acid esters of glycerol and they are the major constituent of adipose tissue. A triacylglycerol molecule is an ester of three fatty acids and a trihydroxy-alcohol, called glycerol (Figure 2.1). The three carbon atoms of glycerol are designated as α, β and α', or more commonly as 1, 2, 3; the β carbon is chiral.

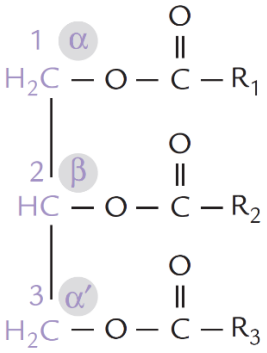

Figure 2.1. Basic composition of a triacylglycerol. The glycerol backbone is coloured. The fatty acids esterified with the second carbon (R2–COOH) and the third carbon (R3–COOH) are usually unsaturated fatty acids, whereas (R1–COOH) is a saturated fatty acid. On hydrolysis with aqueous acid or alkali the triacylglycerols are split into glycerol and a mixture of fatty acids (Puri 2014).

Enzymic hydrolysis usually occurs with some degree of regiospecificity. Most commonly this involves deacylation of the a-positions (sn-1 and sn-3) with no reaction occurring at the sn-2 position so that the products are fatty acids from the α positions and 2-monoacylglycerols (Puri 2014).

This selective de-acylation, occurring with a lipase, (designated lipolysis) is an important step in the metabolism of triacylglycerols. Pancreatic lipase, the main lipid digestive enzyme, catalyzes the removal of fatty acids only from positions 1 and 3 (Gunstone 2012; Puri 2014).

2.1.2 Phospholipids

These are amphipathic molecules with aliphatic tails (hydrocarbon) and polar heads. They are major components of the biological membranes. The polar heads of the phospholipid tend to extend to exterior, whereas the nonpolar tails move towards the interior,

where they associate with other nonpolar constituents of the membrane including glycolipids, cholesterol and some proteins (Puri 2014).

2.1.3 Sphingolipids

The component units of sphingolipids are fatty acids, often including 2-hydroxy acids, bound as amides to long-chain amines. These amines have also two or three hydroxyl groups, one of which is linked to one or more sugar units for every phosphodiester unit.

Although structurally different, the phosphor-glycerides and sphingolipids resemble each other in that they both contain two long chains and a polar head group and are conveniently located in lipid bilayers (Gunstone 2012).

2.1.4 Glycolipids

The glycolipids are formed by one mono or oligosaccharide bound to every lipid (ceramide). Depending on the nature of the carbohydrate component attached, four types of glycolipids are recognized: cerebrosides, sulfatides, globosides and gangliosides (Puri 2014).

2.1.5 Steroids

Steroids are complex molecules consisting of four fused carbon rings. There is a phenanthrene nucleus made of three six-membered rings and a cyclopentane. The steroid nucleus in its fully saturated form is known as cyclopentanoperhydrophenanthrene.

The alcohol derivatives of steroids, in which one or more OH groups are present in the steroid nucleus, are termed sterols.

In animal tissues, cholesterol is the major sterol. It has a single polar head group (hydroxyl group) at the C-3 position and the rest of the molecule is nonpolar. Functionally, cholesterol is a very important molecule in being a precursor for three useful compounds: the bile acids, the steroid hormones and vitamin D. Cholesterol is an essential component of all biological membranes (Puri 2014).

2.1.6 Waxes

Waxes are the esters of fatty acids with long chain alcohols.

They come from animal (beeswax, wool wax, sperm whale oil, orange roughy oil) and from vegetable sources (candelilla, carnauba, rice bran, sugar cane, jojoba) and may be solid or liquid. In general, the term wax is applied to water-resistant materials made up of hydrocarbons, long-chain acids and alcohols, the esters derived from these and ketones (Gunstone 2012; Puri 2014).

2.2 Fatty acids

Fatty acids, both free and as part of complex lipids, play a number of key roles in the metabolism of major metabolic fuel (storage and transport of energy), as essential components of all membranes and as gene regulators.

The fatty acid molecule consists of a long hydrocarbon chain with a polar carboxyl group at its end.

The fatty acids with hydrocarbon chains containing one or more double bonds are called unsaturated fatty acids, whereas those lacking any double bonds are referred to as saturated fatty acids (Figure 2.2).

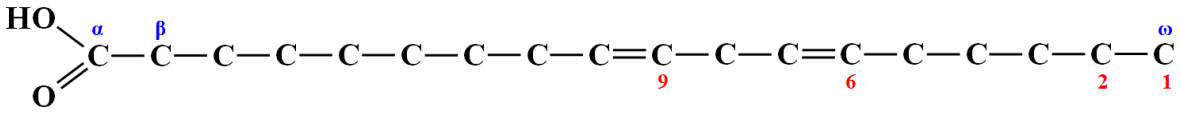

Figure 2.2 Exemple of a fatty acid (18:2n-6).

Fatty acids show several function such as components of membrane, source of cellular fuel, energy reserve within the adipose tissue in the form of triacylglycerols and they take part in the composition of hormones and lipids (derivatives of fatty acids) (Puri 2014).

2.2.1 Nomenclature

A chemical name must identify and describe its chemical structure unambiguously. This is done by using IUPAC nomenclature (or systematic).

There is another way of naming the fatty acid, the common nomenclature, which includes circumstantially assigned names; for example, source names are widely used e.g the name oleic acid stem from olives oil because this is the principal fatty acid present in olives oil. Widely used is also the nomenclature based on the chemical structure. The first number indicates the number of carbon atom, the second number indicates the number of double bonds and the last number is a number of the carbon of the first terminal double bond. For example, Linoleic acid or (9Z,12Z)-9 12-Octadecadienoic acid, becomes C18:2Δ9.

The Greek letters omega (ω) and delta (Δ) are sometimes used with special significance in naming fatty acids. Omega (ω) is often used to indicate how far a double bond is from the terminal methyl carbon irrespective of the chain length. The “n” system is analogous to the “ω” naming system. Delta (Δ), followed by a numeral or numerals, is used to designate the presence and the position of one or more double or triple bonds in the hydrocarbon chain counting from the carboxyl carbon (Chow 2007).

2.2.2 Classification

Fatty acids can be classified in different ways: i) number of carbon, ii) presence or absence of double bonds, iii) number of double bonds iv) position of the first double bond starting to the end of aliphatic chain (carbon ω).

Number of carbon (chain length). The number of carbon in the back-bone of a chain of fatty acid determines its chain length (O’Brien 2008):

- Short-chain: less of 6 carbon atoms
- Medium-chain: comprised between 6 and 12 carbon atoms
- Long-chain: more than 12 carbons atoms.

Number of double bond. The fatty acids can be grouped on the basis of absence or presence of double bonds between their carbon atoms. There are two broad classes termed saturated

and unsaturated; unsaturated fatty acids are further divided into mono- and polyunsaturated (O'Brien 2008).

Saturated Fatty Acids (SFA) do not contain double bonds between carbons; chain contains only single bonds (σ bond) (O'Brien 2008).

Unsaturated Fatty Acids (UFA) have one or more double covalent bonds between adjacent carbon atoms (O'Brien 2008).

Monounsaturated fatty acids (MUFA) have only one double bond (π bond), which can occur in different positions. The most common is known as monoene and has a double bond with the *cis*-configuration. This means that the hydrogen atoms on either side of the double bond are oriented in the same direction. (Rustan and Drevon 2005).

Polyunsaturated fatty acids (PUFA) have more double bonds. Chemical reactivity increases as the number of double bond increases. Double bond in PUFA are conjugated or rather, double-bonded carbon atoms alternate with single bonds carbon (Figure 2.2) (Rustan and Drevon 2005).

2.2.3 Properties

Solubility. Fatty acids are predominantly nonpolar in nature because of the long hydrocarbon chain. This accounts for the insolubility of lipids in water and other polar solvents and the solubility in the nonpolar solvents (Puri 2014).

Melting point. The melting point of fatty acids is dependent on two factors: degree of unsaturation (the melting point falls as the number of double bonds increases) and chain length (the increase in chain length corresponds to a rise in the melting point) (Puri 2014).

Hydrogenation. The double bonds of unsaturated fatty acids can be hydrogenated to form saturated fatty acids (Puri 2014).

Oxidation. Unsaturated fatty acids can spontaneously react with oxygen to form peroxides, epoxides and aldehydes of fatty acid. Oxidation is prevented by antioxidants (enzymatic and non-enzymatic) (Puri 2014).

2.2.4 Metabolism

2.2.4.1 De novo synthesis of fatty acids

Fatty acids are obtained from both diet and *de novo* (new) synthesis. Most fatty acids have multiples of two carbon atoms, because they are synthesized from successive addition of two carbon units (acetyl CoA) in the cytosol. The elongation of the fatty acid chain stops upon formation of palmitate (C16). Further elongation (and desaturation) are carried out by other enzyme systems.

The biosynthetic pathway occurs in 3 stages: transport of acetyl CoA to cytosol; conversion of acetyl CoA to malonyl CoA; reactions of fatty acids (Puri 2014).

2.2.4.2 Catabolism

β-Oxidation. It is the principal pathway for catabolism of fatty acid. It consists of repeated cycles of a series of reactions. Within each cycle, one molecule of acetyl CoA is removed from the carboxyl terminal of the fatty acid. The pathway occurs in three stages: activation of fatty acid in the cytosol, transport of activated fatty acid into mitochondria and standard β-oxidation process (dehydrogenation, hydration and dehydrogenation) in the mitochondrial matrix (Puri 2014).

Mitochondrial β-oxidation. It can smoothly oxidize unbranched saturated fatty acids with an even number of carbons and a chain length up to 18 or 20 carbons. Fatty acids that do not fit this description require additional enzymatic reactions; for instance, methylated fatty acids require α-oxidation initially before they can be beta-oxidized and very long chain fatty acids (VLCFA) are handled initially by peroxisomal oxidation pathway. Therefore, medium chain fatty acids are degraded by ω-oxidation; unsaturated and odd chain fatty acids also require additional processing before they are beta-oxidized (Puri 2014).

α-Oxidation. It is a minor pathway occurring in the endoplasmic reticulum and mitochondria. It removes one carbon unit at a time from the carboxyl end. A methylated fatty acid cannot be β-oxidized without prior modification (Puri 2014).

Oxidation in peroxisomes. Essentially is uses the same reaction sequence as the mitochondrial system, except for the very first reaction, which is catalyzed by an

H_2O_2-producing flavoprotein oxidase. The main purpose of peroxisomal β-oxidation is to shorten very long chain fatty acids, thereby boosting β-oxidation (Puri 2014).

Omega (ω)-oxidation. It is a microsomal system for oxidation of the carbon atom further most remote from the carboxyl group, to obtain a dicarboxylic acid. Medium chain fatty acids mobilized from the adipocyte triacylglycerol stores are oxidized in the liver by ω-oxidation (Puri 2014).

Oxidation of Unsaturated Fatty Acids. The reaction for the oxidation of unsaturated fatty acid is the same of β-oxidation, with an exception: when a double bond is reached, the cycle cannot proceed any further because the intermediate formed at this stage is not a natural substrate for the enzymes of β-oxidation (Puri 2014).

Oxidation of Monounsaturated Fatty Acids. For the oxidation of monounsaturated fatty acids, it is necessary to convert the *cis*-double bond in *trans*-double bond. After the isomerization of the double bond, the β-oxidation can then proceed with the normal pathway of oxidation (Puri 2014).

Oxidation of Polyunsaturated Fatty Acids. The enzymatic difficulty arises due to the presence of two or plus *cis*-double bonds. The initial reaction sequence takes place as in the case of monounsaturated fatty acid. After isomerization and one cycle of β-oxidation, another cycle of reduction and isomerization follows, to convert the second double bound. This cycle repeats until all the double bounds are degraded (Puri 2014).

2.3. Membrane, fatty acids and temperature

Cellular membrane is an important structure of cells that preform a lot of biological function as compartimentalization, extracellular recognition, signal transduction, transport (ions, glucose, ..., etc.) and enzyme catalysis (Yeagle 2001).

2.3.1 Lipid bilayer

All biological membranes are constituted by a structure of two layers held together by propriety of lipids which constitute it (hydrophobic effect).

Since that the biological environment is an aqueous medium, the lipids of membrane self-assembly in a bilayer where the polar heads are exposed on the surface and the hydrophobic tails constitute the core of this structure.

One characteristic of biological membrane is the asymmetry i.e. the composition of lipids that constitute the two layers is different; for example, phosphatidylcholine shows a great concentration in the external surface and phosphatidylethanolamine is present in major quantity in the internal surface (Yeagle 2001).

2.3.2 Effects of temperature on biological membranes

The temperature is a very important factor for biological organism because it determines the rates of chemical reactions and changes the kinetics, the equilibria and the disruption of the noncovalent interactions in biological structures. The effects of temperature on the membrane cell are complex and determine the state of the membrane (sol-gel), the molecular motion of membrane constituents, and can significantly perturb the membrane function (Hazel 1990).

2.3.3 Thermal perturbation of membrane structure and function

Thermal perturbation is when the body temperature in poikilothermy animals changes and corresponds with parallel perturbation of biological membrane. At physiological temperatures, gauche rotamers (rotations about carbon-carbon single bonds) freely propagate up and down the length of the fatty acyl chains, which results in a relatively fluid, disordered liquid-crystalline phase (Figure 2.3). When temperature drops below the physiological range, acyl chains, at some defined point (transition temperature, Tm) increase pack efficiently to form a highly ordered gel phase (Hazel 1995).

Conversely, when temperature exceeds the physiological range, some lipids (most notably phosphatidylethanolamine), assume the inverted hexagonal phase, which results in a loss of bilayer integrity. Finally, even in the absence of lipid phase transitions, rising temperature increases the rate and the extent of acyl chain motion (Hazel 1995).

A variation in temperature involves a change in the state of the lipid phase, which determines a variation of the membrane proprieties, such as a reduction of the activity of membrane-associated enzymes, an increase or decrease of the permeability to cations and water, and a variation of the diffusion of protein and other molecules in membrane (Hazel 1995).

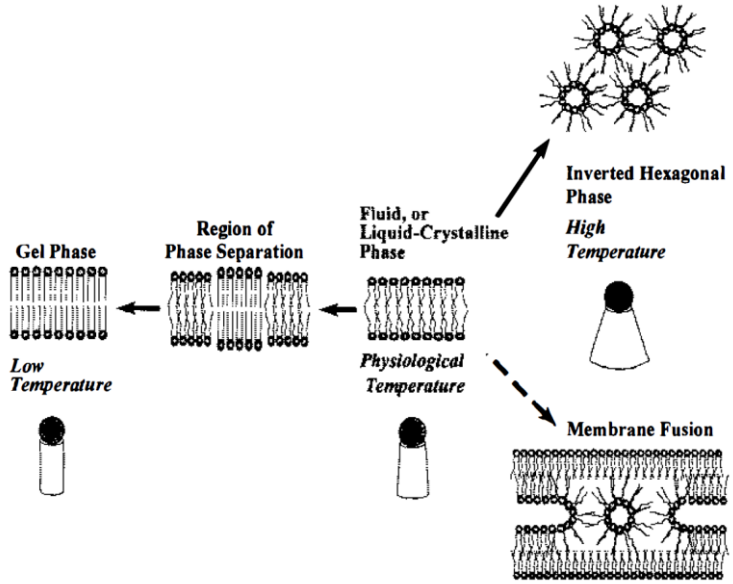

Figure 2.3. Solid arrows indicate the effects of either a rise or drop in temperature on the phase behavior and molecular geometry of membrane phospholipids. The physiological temperature refers to the temperature at which an organism is either adapted or acclimated. The dashed arrow illustrates the presumed involvement of the inverted hexagonal phase in membrane fusion (Hazel 1995).

2.3.4 Membrane remodeling: the basis of thermal adaptation

When there is a change in environmental temperature, the poikilothermic organisms must restructure the chemical composition of lipids in biological membrane to keep appropriate physical properties of membrane. (Hazel 1990, 1995).

In general principle, change in lipid composition occurs when there is a change in temperature, such as changes in the PUFA concentration (especially long chain PUFA) and/or in the content of SFA, little changes in the total content of monoenes and dienes, and changes in the composition of phospholipids (Hazel 1995, 1979).

3 Fishes

Until now there are about 30,000 species of fishes and most of them are bony teleosts (96%) followed by cartilaginous elasmobranchs. All other fish groups have insignificant numbers of species (Bone and Moore 2008).

3.1 Distribution and morphology of teleostei

From their evolution since the Cretaceous, teleosts (Figure 3.1) have colonized all habitats and they were adapt into many different niches. Teleost are characterized from a skeletal element well calcified. Naturally, there are exceptions, such as for example fishes living in the deep-sea, which had a low rate of calcification of skeletal to save weight (Bone and Moore 2008).

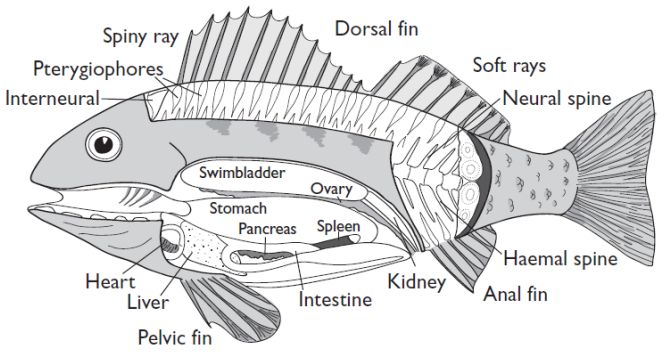

Figure 3.1. General features of a teleost (*Perca*) (Bone and Moore 2008).

3.2 Muscle

The principal chemical components of muscle are water, protein and lipids (accounting together for about 98%) and in minor parts, carbohydrates, vitamins and minerals, but they develop an important biochemical role.

The fish muscles are divided by thin connective tissue membranes, known as mycommata, and into segments called myotomes. Each myotome is composed of muscle fibers running parallel to the long axis of the fish. Each fiber is surrounded by a membrane called sarcolemma, which contains thin collagenous fibrils. These fine fibrils merge with

mycommata at the myotome-mycommata junction. In contrast, the muscle mass in terrestrial animals is elongated and is tapered into a tendon.

The myofibrils are segmented into sarcomeres, which are composed of thin and thick filaments, showing alternate arrangements of anisotropic (A) and isotropic (I) bands bordered by Z-lines.

Fishes present two different type of muscles (white and dark). Dark muscles are utilized for continuous swimming motion and are located along the lateral line of most fish; they contain more mitochondria and have higher concentrations of lipids, glycogen and nucleic acids than white muscles. White muscle are utilized for fast and powerful movements but since they have an anaerobic metabolism they do not allow for prolonged swimming; they show higher concentrations of ATPase activity, glycolytic acids, and water than dark muscles (Venugopal and Shahidi 1996).

3.3 Liver

Liver is a very important organ and play a key role in fish physiology (Figure 3.2), both in the anabolism and in the catabolism of a lot of molecules (carbohydrates, proteins, lipids), in the detoxification, and in the glycogenolysis. Liver in fishes plays a fundamental role in vitellogenesis.

The fish liver is a principal target for many biological and environmental effects (Brusle and Anadon 1996).

There are two important characteristics in the physiology of liver in fish.

The first characteristic is that fishes are poikilothermic vertebrates: a variation in the ambient temperature determines substantial changes in their metabolism. Fish are especially susceptible to environmental variations and respond more sensitively to pollutants than numerous mammals.

The second characteristic is that there are differences in the liver structures between males and females and between immature and mature fish.

Based on these characteristics, liver is then a very interesting model to study the influence of environmental factors on hepatic structures and functions (Figure 3.3) (Brusle and Anadon 1996).

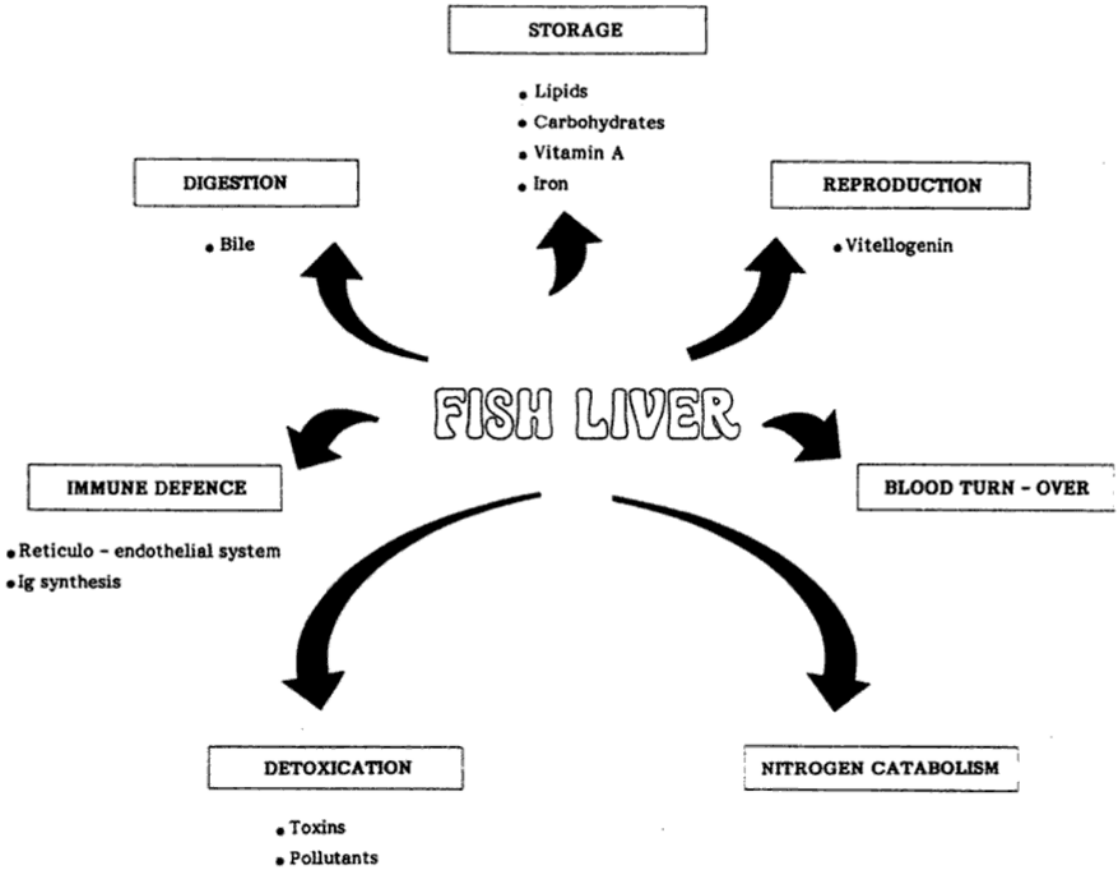

Figure 3.2. Prominent function of fish liver (Brusle and Anadon 1996).

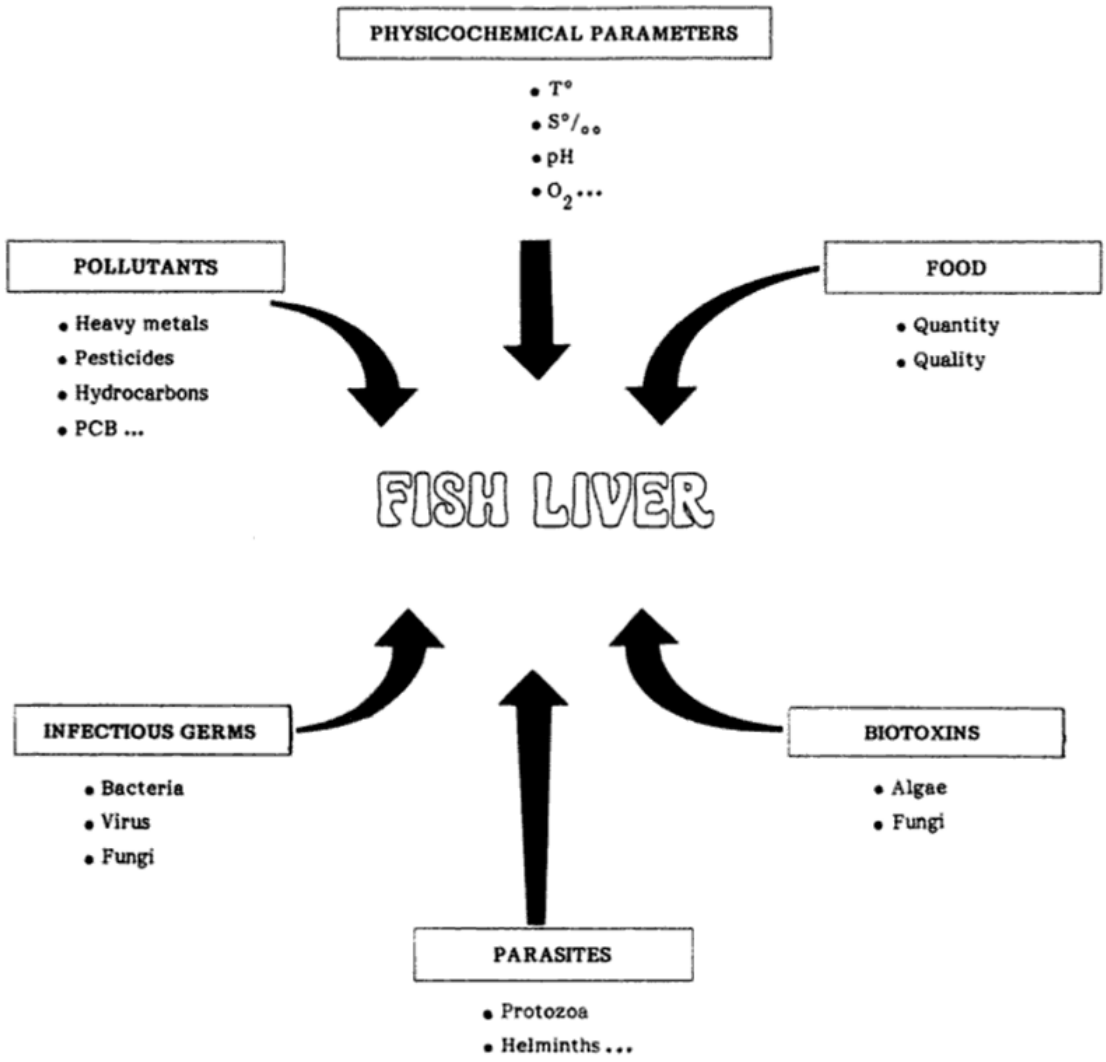

Figure 3.3. Different stressors towards the fish liver (Brusle and Anadon 1996).

3.4 Gills

Gills are an important organ that separates external medium from the blood, across which gaseous exchange takes place. The respiratory surface is generally very much folded to increase the surface of exchange.

The gills have an abundant flux of blood and in general in sites of gaseous exchange blood passes in the lacunar spaces between the pillars or trabeculae so that exchange with the external medium can take place across both sides of the respiratory lamella. The flow of water over such surfaces is nearly always unidirectional.

The gills of vertebrates are formed as a series of paired pouches in the lateral walls of the pharynx which open externally by gill slits. Adjacent pouches are separated by interbranchial septa containing skeletal elements that support the gills. In fish, the interbranchial septa are supported by a series of visceral arches, each consisting of several skeletal elements forming an incomplete hoop within each arch on the pharyngeal side. Lateral plate mesoderm isolated between the gill slits forms branchial muscles, associated with the visceral skeleton. Behind each gill slit passes the main post-trematic branch (viscero-motor and sensory) of the IXth or Xth cranial nerves, and a smaller pretrematic branch (viscerosensory) passes anterior to the slit.

Afferent filament arteries branch from the afferent branchial artery and convey blood along the edges of the filaments. Branches from these arteries supply blood to the secondary lamellae (Huges and Morgan 1973).

3.5 Lipids in fish

It is possible to divide fishes in two categories, in according to their total content of lipids: fat lean fish (under 5 percent of total lipids) and fatty fish (over 5 percent of total lipids).

Lipids in fatty fish are mostly subcutaneous while in lean fish, they are deposited in the liver, muscle tissue, and in mature gonads.

Marine lipids are composed of triacylglycerols, phospholipids, sterols, wax esters, and some unusual lipids, such as glyceryl esters, glycolipids, sulfolipids, and hydrocarbons. The amounts of lipids in fish may vary from 0.2% to 23.7%, depending upon anatomical position, sex, season, and diet, most of the variations being in the triacylglycerol content while phospholipids show much less variation.

Fatty acids are usually composed of monoenoic (15-40%), saturated (20-35%), and polyenoic (38-51%) acids. Marine fish lipids are highly unsaturated, the ratio of polyenoic to saturated acids being 3:1, while in freshwater fish, the ratio is 1:1. Marine fish contain concentration of n-3 and n-6 higher than freshwater fish.

The phospholipids of tropical fish are more saturated than those from cold water (Venugopal and Shahidi 1996).

3.6 Metabolism of fatty acids in fish

Vertebrates lack the Δ12 and Δ15 desaturases and can not synthesize respectively linoleic (LIN, 18:2n-6) and α-Linolenic acids (18:3n-3, ALA) from oleic acid (18:1n-9). Therefore, these polyunsaturated fatty acids are essential dietary components. However, 18:2n-6 and 18:3n-3 can be elongated and desaturated in organisms to synthesize arachidonic (ARA, 20:4n-6), eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:5n-3) acids (Figure 3.4). These particular fatty acids are called essential fatty acids (EFAs) and must be introduced with the diet. In addition, fish vary considerably in their ability to convert EFAs between the different species. The EFAs requirement of the fish is thus related to their ability to convert these fatty acids (Arts et al. 2009; de Silva and Anderson 1994).

With one exception, the reactions occur in the smooth endoplasmic reticulum with the same enzymes acting on both n-3 and n-6 fatty acids, although the affinity of the enzymes is generally higher for the n-3 series.

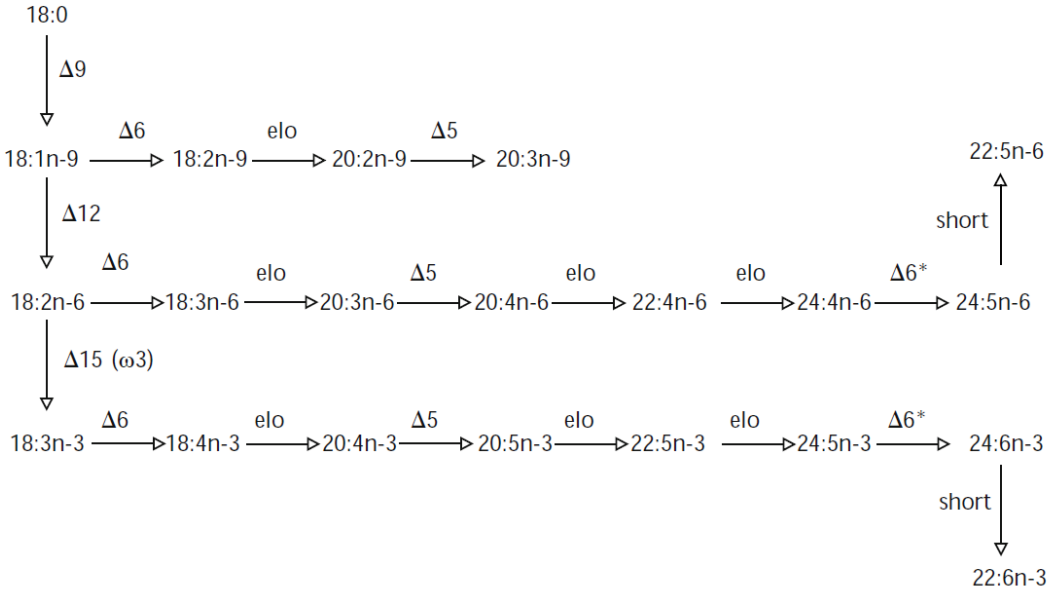

Figure 3.4. Pathways of biosynthesis of 20 and 22 highly unsaturated fatty acids from n-3, n-6 and n-9 C18, precursors. Δ5, Δ6, Δ6*, Δ9, Δ12. Δ15 (ω3), fatty acyl desaturases; elo, fatty acyl elongases; short, chain shortening (Arts et al. 2009).

Like all vertebrates, PUFA are essential dietary components for fish. However, the biologically active and physiologically important PUFA in fish are the highly unsaturated fatty

acids (HUFA) such as ARA, EPA and DHA, since LIN and ALA, have no specific or unique metabolic role in themselves.

Freshwater fish have sufficient Δ6- and Δ5-desaturase and elongase to produce ARA, EPA, and DHA from their precursors, if they are present in the diet, but marine species have limited elongase and desaturase capability that requires an assumption of these EFAs (Holt 2011)

In marine fishes their natural diet is rich in EPA and DHA and they do not have the necessity to synthesize these fatty acids. In freshwater fish, the conversion of ALA to DHA may be more necessary because their natural diets (particularly their invertebrate prey) is poor in n-3 HUFA.

Although the Δ6 desaturases were cloned in marine fish, the activities measured in hepatocytes and enterocytes from cod and sea bass were very low (Arts et al. 2009). The expression of Δ5 desaturase and elongase genes in liver of marine fish, determined by Q-PCR, were positively and negatively correlated with dietary intake of ALA and n-3 HUFA, respectively (Arts et al. 2009).

3.7 Lipid digestion and absorption in fish

In general, digestion, absorption, and the transport in fish are similar to that in mammals. Nonetheless, the complexity of the intestinal tract and marked anatomical differences between different species of fish, including highly variable numbers of pyloric caeca and the presence or lack of a pancreas, have made the study of digestion in fish very complicated (Tocher 2003).

Digestion. Lipolytic activity in fish is generally greatest in the proximal part of the intestine and the pyloric caeca (if present), but it can extend into the lower parts of the intestine with the activity decreasing progressively. Exceptions occur, however, as lipolytic activity is higher in the distal part of the intestine in turbot (*Scophthalmus maximus*) and plaice (*Pleuronectes platessa*) (Tocher 2003).

The pancreas or hepatopancreas is generally assumed to be the major source of digestive lipase enzymes in fish as it is in mammals. However, digestive lipases may also

be secreted by the intestinal mucosa, as several studies have found high lipase activity in mucous layers or intestinal segments of many fish species (Tocher 2003).

Triacylglycerol is a major lipid class in the diet of marine fish and it is generally the predominant lipid class in the diet of freshwater fish.

The existence of a lipases dependent to bile salts was demonstrated in teleost fish. A lot of studies suggest that the main triacylglycerol lipases are also a bile salt-activated lipase.

Although studies are difficult to compare due to the wide range of substrate preparations and experimental conditions used, overall the data suggest that a bile salt-activated lipase is the main lipolytic enzyme in teleost fish, whereas the existence of a pancreatic lipase-colipase system is less certain (Tocher 2003).

Phospholipids (particularly phosphoglycerides) are a substantial part of the dietary lipid in fish but there are relatively few studies on the intestinal digestion of phosphoglycerides. It is supposed that the mechanisms in fish are generally similar to those in mammals (Tocher 2003).

It is not clear if fish possess a specific wax ester hydrolase because no specific enzyme has been founded. In addition, the study on substrate of triacylglycerol lipases in fish suggests that this enzyme activity hydrolyzes wax esters, although at a much slower rate than triacylglycerols.

Cholesteryl ester hydrolase has been reported in a few study but the exact enzyme responsible of this activity was not founded (Tocher 2003).

Absorption. The main products of lipid digestion in fish are free fatty acids produced by lipolytic action on all major lipid classes. The absorption of the products of lipid digestion have not been studied extensively in fish, but the basic physical processes, including bile-enhanced emulsification and transport of the hydrolyzed products, are assumed to be generally similar to that in mammals. Thus, the main hydrolytic products are solubilized or emulsified in bile salt micelles, followed by diffusion to the intestinal mucosa where uptake into the enterocytes occurs. Absorption generally proceeds at a much slower rate in fish compared with mammals, because of lower body temperature that strongly influences the rate of nutrient digestibility. As in mammals, lipid absorption in fish occurs predominantly in the proximal part of the intestine, where there is the highest lipolytic activity. However, lipids

can be absorbed along the entire length of the intestine, even if it occurs in diminishing amounts.

In the enterocytes, the absorbed free fatty acids are re-esterified with glycerol, to obtain partial acylglycerols, and lysophospholipids to reform triacylglycerols and phosphoglycerides (Tocher 2003).

Extracellular Transport. Lipids are distributed from the intestine to other tissues in the form of lipoproteins. The lipid load and the degree of unsaturation affect lipoprotein production, with high dietary lipid and PUFA leading to the production of larger chylomicrons, whereas high dietary saturated fatty acids, results in the production of smaller VLDL particles.

In fish, most of the intestinal lipoproteins are transported via the lymphatic system before appearing in the circulatory system and being transported to the liver. However, a portion of intestinal lipoproteins may be transported directly to the liver via the portal system.

The type of lipoproteins that is possible to find in fishes are the same of mammalian: chylomicrons, VLDL, low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The proportion of total lipids and core lipids, the major neutral lipids triacylglycerol and steryl esters, decreases from chylomicrons through VLDL to LDL and HDL, whereas the proportion of surface components such as phospholipids, free cholesterol, and protein increases (Tocher 2003).

Intracellular transport. The intracellular transport of fatty acids is mediated by fatty acid binding proteins that are tissue specific. Several fatty acid binding proteins were founded and studied in different fishes and similarities were found with those of mammals. The studies demonstrated that in general the intracellular transport of fatty acids in fish is essentially the same as that in higher vertebrates (Tocher 2003).

3.8 Role of fatty acids in fish

In general, the principal roles of fatty acids in the fish are: i) source of metabolic energy, ii) structural components of cell membranes (phospholipids and glycolipids in membrane), and iii) precursors of bioactive molecules. In particular, DHA, EPA and ARA are important fatty acids with physiological functions; these fatty acids are critical components of cellular membrane (phospholipids) that facilitate key intramembrane reactions and processes

(enzymatic or non-enzymatic) for the production of eicosanoids molecules. For example, ARA is the main substrates for the production of eicosanoids.

Eicosanoids are substances physiologically active (local hormones) with autocrine and paracrine functions that regulate a wide array of cellular pathways and cascades. EPA and ARA are competitive precursors for eicosanoid synthesis, but the eicosanoids produced by EPA are frequently considered less biologically active than those produced by ARA. The roles of eicosanoids in fish are quite different and include modulation of reproduction, hormone release, cardiovascular and neural function, osmoregulation and immune function.

The long chain PUFA are able to modulate the transcription of genes involved in lipid homeostasis and the eicosanoids derived to ARA e PUFA modulate the transcription of genes of lipid metabolism (Holt 2011).

3.9 Antarctic fish

The evolution of Antarctic fishes in a cold marine environment (-1.9 °C) have entailed a variety of specializations of the biochemical and physiological processes. Streamlining of some biochemical processes through narrowing of thermal tolerance ranges and reduction in the diversity of synthetic products are also characteristic features of molecular adaptation in Notothenioids (an order of Perciformes that includes Antarctic and sub-Antarctic fishes). One of the main characteristics of their biochemistry is the synthesis of freezing-point depressing molecules (antifreeze molecules), which protect the organism from ice crystal formation in a non-colligative way (Colella et al. 2000). Another peculiar characteristic of these fishes is the reduced level of hematocrit (hemoglobin concentration in blood), with the extreme case represented by the family of Channichthyidae, which completely lack erythrocytes and hemoglobin. The lost or the reduction of hemoglobin concentration is an example of this adaptation to cold, because the oxygen saturation and its concentration in polar seawater is about 1.6 times higher respect water at 20°C (Colella et al. 2000; Eastman 1993).

Other peculiar and important characteristics of these fishes are: i) their preferential utilization of the lipid metabolic pathway, ii) high mitochondria density, iii) high content of intracellular neutral lipid droplets, that increase oxygen solubility in cell and iv) the increase

of mitochondrial oxygen consumption (Colella et al. 2000; Lund and Sidell 1992; Machado et al. 2014; Sidell 1998)

The success of Notothenioids to survive in the Antarctic habitat involves, among other things, adaptive modifications of proteins and membrane lipids, because only the conservation of their biochemical characteristics and physiological state allows physiological processes to proceed at the low temperature of the Antarctic sea (Eastman 1993). The property of poikilothermic animal's cells to adjust the physicochemical characteristics of cells membrane when there is an environmental temperature change is called homeoviscous-adaptation of membrane fluidity. This process that was described for the first time by Sinensky in *Escherichia coli* in 1974 (Farkas et al. 1988; Hazel 1990; Lahdes et al. 2000). The modification of lipid composition is an important factor to maintain the correct physiological function of cellular membrane at extremely low temperatures. The principal strategy of adaptation is a modification of the lipid microenvironment, which controls the activity of the protein and the synthesis of new isoenzymes. Furthermore, lipids composition of Antarctic fishes show elevated levels of MUFA and UFA in membrane and lipid deposits (Eastman 1993; Hazel 1990; Logue et al. 2000; Palmerini et al. 2009).

3.9.1 Warm acclimation response in Antarctic fishes

Fishes of the suborder Notothenioidei comprise the largest fraction of fishes endemic to Antarctic waters (Eastman 1993); they live permanently at low temperatures (about -1.8 °C) an some species are considered highly stenothermal, being highly specialized to survive at low temperatures (Clarke 1991; Eastman et al. 1990; Portner and Knust 2007). Several studies have aimed to explore the warm acclimation response in Antarctic notothenioid fishes, such as *Notothenia coriiceps* and/or *Notothenia rossii* (Forgati et al. 2017; Klein et al. 2017; Machado et al. 2014; Mueller et al. 2014; Souza et al. 2018; Strobel et al. 2013), *Pagothenia borchgrevinki* (Carney Almroth et al. 2015; Franklin et al. 2007, 2001; Lowe et al. 2005; Podrabsky and Somero 2006; Ryan 1995; Seebacher et al. 2005), or other species of fishes, such as the icefish *Chionodraco rastrospinosus* (Mueller et al. 2014) or the eelpout *Pachycara brachycephalum* (Windisch et al. 2014). These studies demonstrated that some species are more tolerant to warmer temperatures arising from climate change and that fishes did not lose the capacity to respond to environmental changes, although they present a narrow window of thermal tolerance.

The Notothenioid teleost *Trematomus bernacchii* (Boulenger, 1902) is one of the most abundant fishes in Antarctic seawater (Near and Cheng 2008) and, for this reason, it is often used as a reliable bio-indicator for environmental studies (Barbaro et al. 2016; Ghosh et al. 2013; Illuminati et al. 2010). Starting from Somero and DeVries (1967), several studies have focussed on the thermal acclimation response of *T. bernacchii*, considering numerous parameters, such as antioxidant defence system (Enzor and Place 2014), transcriptome wide analysis and gene expression (Buckley et al. 2004; Huth and Place 2016; Tolomeo et al. 2016), oxygen consumption and cardiorespiratory performance (Jayasundara et al. 2013; Weinstein and Somero 1998), temperature survival and critical thermal maximum (Bilyk and DeVries 2011; Podrabsky and Somero 2006), physiological performances (Davis et al. 2018; Sandersfeld et al. 2015), cortisol levels (Hudson et al. 2008) and cellular apoptosis (Sleadd 2014). Moreover, fatty acid composition of tissues was explored in *T. bernacchii* exposed to thermal shock. Malekar et al. (2018) demonstrated that a thermal acclimation at 6 °C resulted in an increase of saturated fatty acids and a decline in unsaturated fatty acids in membrane of liver tissue, showing a homeoviscous response to maintain the proper membrane functionality.

After more than 50 years of research focused on the adaptation of Notothenioid to climate warming, there are still many questions that remain to be answered (Beers and Jayasundara 2015). This study provides important novel information on the acclimation of Antarctic fishes to a thermal shock, and it may help to better understand the response of the Antarctic fish to rising temperatures.

4 Fatty acids as biomarkers

A biomarker is a compound that can be uniquely identified and is metabolically stable (Dalsgaard et al. 2003).

Several studes in the last century demonstrated that it is possible to use fatty acids as biomarkers, for example to follow trophic interactions and to trace the flow of energy in the aquatics ecosystems, or to study the effect of temperature and diet on organisms (Alfaro et al. 2006; Copeman et al. 2013; Dalsgaard et al. 2003; Könneke and Widdel 2003; Mourente et al. 2015; Sajjadi and Eghtesadi-Araghi 2011). A particular fatty acid profile, or a specific fatty acid, can be attributed to an individual or groups of organism (algae, higher plant, bacteria, marine fauna, etc.) and thanks to the different fatty acid composition, it is possible to use FAs as biomarkers (Bianchi and Canuel 2011; Dalsgaard et al. 2003; Mourente et al. 2015; Sajjadi and Eghtesadi-Araghi 2011)

Microbial community. FAs are a source of organic matter in aquatic ecosystem. The distinct structures of fatty acids make them useful as biomarkers to characterize microbial community and the sources of sedimentary organic matter in sediment of aquatic environment. Furthermore, fatty acids are used as biomarkers because they are associated with specific eukaryotic and prokaryotic taxa, with differences in FA profiles increasing with phylogenetic distance (Poerschmann et al. 2012).

Sediments. The concentration of FAs in sediments is closely related to the latitude of sediments, and their distribution reflects intense biomass and biological activity. For example, Huang et al. (2015) reported that the total concentrations of FAs of samples around hydrothermal areas were significantly higher than samples away from hydrothermal areas, indicating intense primary production and large biomass in the hydrothermal areas, and suggesting a close relationship between hydrothermal activity and Total FAs of samples. Several fatty acids (e.g., 15:0 and 16:1n-7), which are signature biomarkers for sulfur-metabolizing bacteria, show the same distribution trend as Total FAs of samples, further highlighting the close relationship between fatty acid content and hydrothermal activity and/or hydrothermal communities.

Particulate organic matter. Seasonal studies on fatty acid composition of particulate organic matter in aquatic environment have shown strong temporal patterns associated with community composition of phytoplankton and bacteria, nutrient availability, light intensity,

and proportion of fresh to older material (Leu et al. 2006; Mayzaud et al. 2013; Parrish and Thompson 2005; Reuss and Poulsen 2002).

Sea-Ice algae. Fatty acid can be used as biomarker to determine the food quality of sea ice algae. (Fahl & Kattner, 1993; Leu et al., 2010). Microalgae living in-or attached to the bottom of sea ice represent an important contribution to primary production in marine ecosystems at high latitudes. The fatty acid composition of algae depends strongly on their physiological state and, hence, the environmental conditions they are exposed to. For sea ice microalgae, growth conditions are intrinsically linked to the physical structure of the sea ice and overlying snow cover, and their seasonal changes (Leu et al., 2010). It is known e.g. that light conditions proved to be decisive in determining the nutritional quality of sea ice algae, and the irradiance was negatively correlated with the relative amount of PUFAs (Leu et al. 2010).

From the total lipid composition of sea ice algae, it is possible to characterize algal populations. It is well established that phytoplankton groups contain similar fatty acids but, a characteristic fatty acids profile corresponds to a specific type of algae. For example, it is known that the fatty acid composition of cultured diatom species is dominated by the 16:1 and 20:5 fatty acids. The same result has been found for sea-ice diatoms and natural phytoplankton blooms dominated by diatoms (Fahl and Kattner 1993). In addition, it is possible to follow the different phases of the bloom, for example high concentrations of fatty acids such as 14:0, 16:4n-1 and 20:5n-3 have been reported during the phytoplankton bloom, whereas 18:0, 18:1n-9, 18:2n-2 and 18:4n-3 were abundantly detected during the post-bloom of phytoplankton in the east coast of Canada (Pokhrel et al. 2015).

Ice core. FAs in ice core are closely related to past climatic changes and their ice core profiles can be employed as a proxy to assess the past changes in marine and terrestrial emissions on multidecad-to-centennial time scales. Fatty acids indeed can be deposited over ice sheet and be stored in ice for several hundred years or more. For example, lower molecular weight fatty acids, LFAs (from 12:0 to 19:0) originate from marine phytoplankton, and a reduced concentration in ice core could be associated to a depressed emission of marine derived fatty acids, probably due to the extension of sea ice coverage. In contrast, the high concentration of LFAs may be associated with an enhanced phytoplankton productivity in the open ocean due to the retreat of sea ice and an enhanced emission of fatty acids via bubble bursting processes from sea surface micro-layer. So, higher spikes of fatty acids could be caused by global warming (Kawamura et al. 1999, 1996; Pokhrel et al. 2015).

Aerosol. FAs have been reported as major lipid class compounds in atmospheric samples such as continental and marine aerosols (Simoneit 1977; Simoneit and Mazurek 1982), rain and snow (Kawamura and Kaplan 1986). They originate from terrestrial higher plant waxes, soil particles and marine organisms (Gagosian 1986; Kawamura 1995; Saliot et al. 1991). Chain length distributions of fatty acids have been successfully used as biological tracers to demonstrate long range atmospheric transport of terrigenous materials over the Pacific and Atlantic Oceans (Gagosian 1986; Kawamura et al. 1999; Simoneit 1977) and to evaluate the biogenic contribution to urban aerosols and rainwaters (Kawamura and Kaplan 1986; Simoneit et al. 1991).

Moreover, fatty acid can be used as biomarker to identify the origin of aerosol in the atmosphere. In the marine atmosphere, for example, LFAs, dominated by 14:0 (myristic acid), 16:0 (palmitic acid) and 18:0 (stearic acid), are known to be present as particles. These LFAs originate from marine biota. The release of LFAs to the atmosphere would occur with the bubble bursting process at the ocean surface and it seem that organic surface active materials could be ejected into the atmosphere with sea salts and be abundant in the marine air (Mochida et al. 2002). These molecules are released from the ocean surface to the atmosphere with a seasonal variation of the sea-to-air flux. In general, it is possible to associate LFAs, originating from marine biota, and higher molecular weight fatty acids (HFAs, C_{20}–C_{32}) originating from plant wax and soils at terrestrial source. From LFA/HFA ratio, it is possible to identify the origin of aerosol; lower LFA/HFA ratios are more influenced by terrestrial sources than those with higher ratios (Kawamura et al. 2010; Mochida et al. 2002).

Another application of FAs as biomarkers is the use of fatty acids profiles as indicators of stress from contaminants. Contaminants cause alterations in the lipid profiles which may be related with inhibition of several biological processes which concern the fatty acids, principally the biosynthesis of lipids (Gonçalves et al. 2016) and their oxidative damage due to lipid peroxidation (Ferreira et al. 2010).

In general, fatty acid can be used to obtain qualitative information, but it is difficult to extrapolate quantitative contributions from specific sources, because lipid content varies between different tissue, changes in life cycle (response to morphological and physiological changes), and environmental conditions (temporal and spatial gradients) (Bianchi and Canuel 2011).

5 Quantification of fatty acids in the muscle of Antarctic fish *Trematomus bernacchii* by gas chromatography-mass spectrometry: optimization of the analytical methodology

5.1 Abstract

This work presents data on the quantification of fatty acids (FAs, in terms of mass unit *per* tissue weight) in the muscle of *Trematomus bernacchii*, a key species in Antarctica, often used as bioindicator for contamination studies. Modifications in fatty acids content should be considered a useful biomarker to study how contaminants affect Antarctic biota. The few published studies on the quantification of FAs in muscle of *T. bernacchii* have focused on percentage of individual FAs on total lipids. To perform the quantification of fatty acids, we used an analytical method based on a fast microwave-assisted extraction of lipids from a lyophilized sample, a base-catalyzed trans-esterification of lipid extract to obtain Fatty Acids Methyl Esters (FAMEs), and a separation and identification of FAMEs by gas chromatography-mass spectrometry. With the optimized and validated method, a fast and accurate separation of FAMEs was performed in 43 min. The linearity was checked up to about 320 μg mL^{-1}; limit of detection and limit of quantification are in the range 4-22 μg mL^{-1} and 13-66 μg mL^{-1}, respectively. The optimized method showed a good accuracy and precision. Major fatty acids were 14:0, 16:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-9, 20:5n-3 and 22:6n-3. Quantified FAs compute for about 47 mg g^{-1} tissue dry weight (dw), with 9.1±0.1 mg g^{-1} dw of saturated FAs, 25.5±0.1 mg g^{-1} dw of mono-unsaturated FAs, and 12.2± 0.1 mg g^{-1} dw of poly-unsaturated FAs.

5.2 Introduction

Different contaminants, such as for example polycyclic aromatic hydrocarbons, dioxin-like compounds, metals and pollutants with estrogenic activity, produce oxidative

stress in organisms increasing the production of free radicals (Alves de Almeida et al. 2007; Ferreira et al. 2010; Perrat et al. 2013; Verdin et al. 2006). Lipid peroxidation is a useful parameter to study the response of organisms to oxidative stress, and it is evaluated in general by indirect measures such as antioxidant enzymes variation and/or production of metabolites such as malondialdehyde (Ghosh et al. 2013; Sattin et al. 2015). The interest towards the relationship between environmental pollution or stressful habitat conditions and the biological response of marine organisms based only on lipid and fatty acid (FA) biomarkers, is increasing (Gladyshev et al. 2012; Perrat et al. 2013; Fokina et al. 2013; Fokina 2015; Gonçalves et al. 2016). The total fatty acid composition of a stressed organism, in particular, reflects its trophic condition (Perrat et al. 2013), as well as the adaptation of cell membranes to environmental contaminants (Fokina et al. 2013) or environmental stressful conditions (Fokina, 2015). In general, in these studies, FAs composition was evaluated as percentage of each FA *vs* total FAs content.

In Antarctica, the teleost *Trematomus bernacchii* (Boulenger, 1902), is a key species, and its ecology and reproductive biology have been well characterized (Gon and Heemstra, 1990). It is a sedentary fish that has an approximately 10-year life span; it is widely distributed along the Victoria Land coast and commonly found at depths down to -200 m. Due to its characteristics, it is often used as a good bio-indicator for contamination studies (Bargagli et al. 1998; Borghesi et al. 2008; Cincinelli et al. 2015; Corsolini et al. 2006; Ghosh et al. 2013; Regoli et al. 2005). Very few papers studied the lipid profile in the muscle of *Trematomus bernacchii* (Phleger et al. 1999; Wüerzberg et al. 2011), besides measuring each fatty acid as percentage of total fatty acids. In this kind of measurement, the variation of each single fatty acid depends on the percentage modification of others fatty acids, but it does not identify the absolute variation of any single fatty acid, which would be more useful to better clarify the influence of contaminants or environmental conditions on lipid profile variation. No study so far has quantified fatty acids in the muscle of *T. bernacchii* in terms of mass unit *per* tissue weight.

For the absolute quantification of each individual fatty acid, it is necessary to have an accurate and precise method. In this respect, it is important to underline that marine organisms are usually rich in long chain mono- (MUFA) and poli-unsaturated (PUFA) fatty acids (Holub and Holub 2004; Strobel et al. 2012), which originate from phytoplankton and seaweed in the marine food chain (Visentainer et al. 2007). Moreover, a high percentage of unsaturated FAs is typical of fishes living in very cold waters (Pörtner et al, 2007, Fujisawa

et al, 2010), such as *T. bernacchii*. In order to analyze FAs profile of marine organisms, precisely because of MUFA and PUFA sensitivity to oxidation, it is important to use analytical methodologies that provide a sample treatment ensuring a minimum alteration of lipid profile. In general, the sample treatment involves the extraction of lipids from the matrix and the derivatization of FAs for the analysis with HPLC or GC.

In the last few years, new technologies based on microwave-assisted extraction (MAE) for the extraction of lipids from tissues have received a growing interest, as they allow a minimal sample handling and a fast extraction that exposes the sample to relatively high temperatures for a very short time. Several studies showed that MAE is a well-suited alternative to conventional extraction methods for the extraction of lipids from different types of samples, such as oil, fishes, vegetables, microalgae and food in general (Batista et al. 2001; Mahesar et al. 2008; Ramalhosa et al. 2012; Virot et al. 2007; Virot et al. 2008a; Virot et al. 2008b). Furthermore, it considerably enhances the efficiency of the extraction and is an environmentally friendly technique (Mahesar et al. 2008; Priego-Capote and Luque de Castro 2005).

In order to analyze the FA profile of marine organisms by means of gas-liquid chromatography (GLC), methylation of FAs to obtain more volatile compounds (Fatty Acid Methyl Esters, FAMEs) is the most common procedure to start (Christie and Han, 2010).

The most widely used method to analyze FAMEs rely on GLC coupled to mass spectrometer (MS) or flame ionization detector (FID) (Christie and Han, 2010; Jumat, et al. 2006). For accurate identification and exact quantification of fatty acids, mass spectrometry is necessary because of the combination of mass spectra and retention times (Zhang et al. 2014). Moreover, the selected ion monitoring (SIM) mode often achieves good sensitivity and accuracy for quantitative analysis of target compounds (Thurnhofer and Vetter, 2005). Compared to FID, MS-detector provides an improved selectivity and a higher sensitivity (Vetter and Thurnhofer, 2007).

The aim of the present study was to perform a reliable and accurate identification and quantification (in terms of mass unit *per* tissue weight) of fatty acids in the muscle of *Trematomus bernacchii*. To achieve this goal, we optimized and validated an analytical methodology modified from the literature, based on i) microwave-assisted extraction of lipids from a lyophilized sample, ii) derivatization of lipid extract using $NaOCH_3$, and iii) FAMEs separation and identification by GC-MS. A lyophilization step was introduced before microwave-assisted extraction of lipids from tissue, in order to eliminate water from samples.

Furthermore, GC-analysis required the optimization of the temperature program for the best peak resolution and minimum run time.

This work contributes to provide a valuable tool to answer one of the eighty priority questions proposed for the next two decades about science in Antarctica, *i.e.* how next-generation contaminants will affect Antarctic and Southern Ocean biota (Kennicutt II et al. 2014).

5.3 Material and methods

5.3.1 Sample collection

During the XXVIII Italian Antarctic Expedition (Austral summer 2014–2015) nine sexually mature specimens of *T. bernacchii* (weight 136-333 g, length 22-30 cm) were caught by a fishing rod in Tethys Bay (74°42'052" S, 164°02'267" E) at the depth of approximately 30 m, very close to the ‘‘Mario Zucchelli’’ Station at Terra Nova Bay, Ross Sea, Northern Victoria Land. Fishes were taken to the laboratory, where they were rapidly measured, weighed and dissected. Muscle tissues were isolated, frozen in liquid nitrogen and stored at -80 °C for transport to Italy and analysis.

5.3.2 Extraction of lipids

Fish fillets were minced, homogenized (homogenizer MZ 4110, DCG Eltronic), and divided in aliquots of about 1 g each. Analyses were carried out on three aliquots *per* fish. After sample homogenization, we introduced a freeze-drying process that allows a complete loss of water at low temperature (-20 °C) and at low pressure, preventing FAs peroxidation. Tissues were accurately weighed and freeze-dried (Edwards EF4 modulyo, Crawley, Sussex, England) until constant weight (±0.2mg). Samples were transferred in a Teflon extraction vessel of a Microwave Accelerated Reaction System, MARS-X, 1500 W (CEM, Mathews, NC, USA) with 20 ml of petroleum ether (35-60 °C):acetone (2:1, v/v, Carlo Erba, Milano, Italy), to perform a Microwave-Assisted Extraction (MAE) according to a procedure of Ramalhosa et al (2012). The extract, filtered through Whatman GF/C filter papers (Ø 90 mm, GE Healthcare Life Sciences, Buckinghamshire, UK) filled with anhydrous sodium

sulphate (Carlo Erba, Milano, Italy) and rinsed twice with further 2 mL of a petroleum ether:acetone mixture, was evaporated under laminar flow inert gas (N_2) until constant weight. After drying, the mass of extracted lipids was determined.

5.3.3 Preparation of fatty acid methyl esters (FAMEs)

The lipids extracted were converted to their fatty acid methyl esters (FAMEs) according to a modified method ISO 12966-2:2011 (revision of ISO 5509:2000), using the methyl ester of nonadecanoic acid (19:0, 99.6%, Dr. Ehrenstorfer GmbH, Germany) as internal standard (IS) (Christie, 1989). Briefly, lipid extract was dissolved in 0.5 mL of n-heptane (Baker, Philipsburg, NJ, USA), then 10 µL of 2 M sodium methylate (obtained by methanol, Baker, Philipsburg, NJ, USA, and sodium methoxide for synthesis ≥ 97%, Merck, Hohenbrunn, Germany) were added. After vortexing for 3 min, the solution was centrifuged at 1000 rpm (92.794 g) for 1 min, then the clear solution was neutralized with ~40 mg of sodium hydrogen sulfate anhydrous, extra pure (Scharlau, Sentmenat, Spain). After the salt settled, 100 µL of the upper phase were transferred to a 1-mL vial and diluted with 400 µL of n-heptane for GC analysis. All solvents and reagents were of HPLC grade.

5.3.4 Instrumentation and analytical conditions

GC-MS analyses of FAMEs were performed on an Agilent-6890 GC System coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 µm film thickness) capillary column was used to separate FAMEs. The instrumental parameters were optimized using a 37-Component FAME mix (≥ 99%, Supelco, Bellefonte, PA, USA) and the methyl ester of nonadecanoic acid as internal standard (IS). Initial instrumental conditions were chosen from the Application Note 5988-5871 EN, Agilent, (David, 2003). Data were collected under scan mode for FAMEs identification, and in SIM mode for their quantification. After a solvent delay of 2.0 min, the following fragment ions were recorded: *m/z* 74 and 87 for saturated, *m/z* 74 and 55 for monoenoic fatty acids, *m/z* 67 and 81 for dienoic fatty acids, and *m/z* 79 and 81 for other polyunsaturated fatty acids (Thurnhofer and Vetter, 2006; Zhang et al. 2014). Identification of fatty acids was achieved using the NIST reference mass spectra database (NIST, Mass Spectral Database 02, National Institute of Standards and Technology,

Gaithersburg, MD 2002) MS search 2.0a (NIST,2002, NIST, Ringoes, USA). Retention time and mass spectra of 37-component FAME mix standard were used to confirm the NIST identification of fatty acids in the sample.

The mass fraction of fatty acids in mg g^{-1} tissue dry weight was measured against the IS. Since the detector response is different depending on the FAME, for each fatty acid the response factor (Rf) was calculated using IS as reference substance (Johnson and Saikia, 2009). For each sample, at least three runs were performed on the GC-MS.

5.3.5 Method performance

In order to verify the performance of the modified analytical methodology, quality parameters such as linearity, limit of detection (LOD), and limit of quantification (LOQ), were evaluated. To estimate these parameters, calibration curves were prepared using 6 standard solutions obtained from dilution of 37-Component FAME-mix, with concentrations ranging from 23.6 µg mL^{-1} to 323.7 µg mL^{-1}.

The LOD and the LOQ were calculated according to ICH Q2B (ICH, 2005) using the following equations:

$$\mathrm{LOD} = \frac{3.3 \cdot Sa}{b} \qquad \mathrm{LOQ} = \frac{10 \cdot Sa}{b}$$

where, *Sa* = standard deviation of the intercept of the regression line, *b* = slope of the calibration curve. To test the accuracy of the proposed method, a recovery test was performed fortifying muscle sample with FAME standard solutions at low and high concentrations, corresponding for each fatty acid to standard solutions Std1 and Std6, respectively. Each determination was carried out in triplicate. The precision of the proposed method was evaluated by analyzing one muscle sample 6 times on the same day and once a day for 6 days to evaluate the intra-day and inter-day precision, respectively.

5.3.6 Statistical analysis

Statistical treatment of performance data of the method, in particular the evaluation of linearity range and LOD and LOQ quantification, was performed using Statgraphics Plus software, version 5.1 (Statgraphics, 2000).

5.4 Results and discussion

5.4.1 Lyophilization step

The percentage of water in the muscle of *T. bernacchii* was very high (79±2%) and justified the introduction of a lyophilization step that allows a complete loss of water. It is known, in fact, that water can cause some problems during the extraction of lipids, producing coextractables which complicate analysis; moreover, the presence of traces of water leads to poor recoveries of FAMEs (Basconcillo and McCarry 2008; Juárez et al. 2008). The percentage of lipids extracted with MAE preceded by sample lyophilization, was 1.1±0.2% ww (wet weight), significantly higher ($p < 0.05$) than the percentage obtained whitout lyophilization step, 0.3±0.1% ww (see Table S5.1 in Supplementary Material for complete data-set). To test the accuracy of MAE method, a lipid extraction from muscle sample was performed with the official Bligh & Dyer extraction method (Bligh and Dyer, 1959). The percentage of lipids obtained with the official method was 1.3±0.2% ww (Table 5.1, and Table S5.1 for complete data-set), not significantly different ($p > 0.05$) from the lipid extraction obtained with MAE on freeze-dried samples. Our results are in agreement with literature data (Table 5.1) in terms of lipid percentage *vs* both wet weight and dry weight.

5.4.2 Optimization of GC-MS method

The optimization of instrumental parameters was performed using standard solutions of 37-component FAME mix added with IS (see Table S5.2 for fatty acids nomenclature). In order to obtain a fast, simple and sensitive method for fatty acids detection in muscle of fish, the GC-MS method by Application Note of Agilent (David, 2003) was modified because some overlapping peaks were noted in the chromatogram using the programming temperature set-up by David (2003). In particular, the resolution between C20:3n-6 and

C21:0 (Rs = 0.39), and between IS peak and C18:3n-6 (complete overlapping) was <0.8, i.e. the minimum Rs value required to obtain satisfactory peak separation (see Table 5.2 and Fig. S5.1 in Supplementary Material). Also, *cis* and *trans* isomers of 18:2n-6 overlapped. The separation of FAMEs in the chromatogram was optimized by adjusting the starting temperature of the column oven and the ramp of the temperature program. Table 5.2 shows the optimization of the method due to the resolution of 20:3n-6/21:0, 19:0/18:3n-6, and *cis* and *trans* isomers of 18:2n-6. Examples of chromatograms obtained with different programming temperatures indicated in Table 5.2 are reported in Supplementary Material (Figures S5.1-S5.3). The optimal temperature program conditions for the best resolution and the minimum run time was: start to 100 °C and held for 1min, the temperature was subsequently increased to 150 °C at the rate of 25 °C min^{-1}, to 200 °C at the rate of 5 °C min^{-1} and to 230 °C at the rate of 1 °C min^{-1} (hold = 1 min). The total run time was 43 min, comparable with the run-time of 35 min of Agilent method (David, 2003). Figure 5.1 shows a chromatogram obtained with the optimized temperature program: no overlapping between peaks were noted; only C4:0 was lost but this FA is not present in marine organisms. These conditions also allow the separation of *cis* and *trans* isomers of 18:2n-6.

5.4.3 Performance data

Table 5.3 summarizes performance data of the optimized method, in relation to retention time (t_r), linearity range, LOD and LOQ of each selected FA. The minimum FAs concentrations of the more diluted standard solution are of the same order of magnitude as the LOQ, as expected by ICH method (ICH, 2005). From the analysis of calibration curves, we noted a coefficient $r^2 > 0.95$ for all compounds, indicating that more than 95% of the detector's signal variance was explained by concentration changes. Since the p value is less than 0.05 for all fatty acids, there is a statistically significant linear relationship between detector's signal and FAs concentration at the 95% confidence level in the considered range (min: 23.72 µg mL^{-1}; max: 323.7 µg mL^{-1}, from 0.070 µmol mL^{-1} to 12.2 µmol mL^{-1}). For all fatty acids the intercept is not significantly different from zero ($p > 0.05$). Concerning the slope of the calibration curves, Saturated Fatty Acids (SFA) showed a slope (average value $14 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$) significantly higher ($p < 0.05$) than the slope of MUFA ($6 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$) and PUFA ($6 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$), which confirms the need to use the response factors to be able to quantify FAs using the calibration curve of the internal standard.

The LOD and LOQ values ranged from ~4 μg mL^{-1} to ~22 μg mL^{-1}, and from ~13 μg mL^{-1} to ~66 μg mL^{-1}, respectively. The highest values observed for both limits (21.8 and 66.0 μg mL^{-1}, respectively) were obtained for 17:0, while 14:1n-5 was referred to the lowest values (4.28 and 13.0 μg mL^{-1}, respectively).

In terms of accuracy, the mean recovery of FAs added at low and high concentrations in muscle sample is 96±9% (min–max 81–115%), and 96±7% (min–max 81–111%), respectively (Table S5.3). These recovery values fall within the acceptable range of 80%-120%, indicating that recovery is not influenced by the matrix, and the adopted method is accurate.

Intra-day and inter-day precision was, for major FAs, ≤4% and ≤7%, respectively, indicating a good repeatability of the analyses. For FAs with a percentage <1% *vs* total FAs, intra-day and inter-day precision ranged from 5% to 20%, and from 8 to 25%, respectively (for complete data set, see Tables S5.4 and S5.5 in Supplementary Material).

5.4.4 Fatty acid quantification

Fig. 5.2 shows an example of chromatogram relative to fatty acid composition of muscle of *T. bernacchii*. In general, good selectivity and efficiency was achieved for all compounds within 43 min. Furthermore, no peaks or artefacts interfering with the FAs chromatographic peaks were found.

Table 5.4 shows a complete lipid profile (% *vs* total fatty acids) and fatty acids quantification (in mg g^{-1} dw) of muscle of *Trematomus bernacchii*. Major FAs (percentage > 10%) were 4 (16:1n-7, 18:1n-9, 20:5n-3, 22:6n-3), between 5% and 10% other 4 FAs were found (14:0, 16:0, 18:1n-7, 20:1n-9), and between 1% and 5% only 3 FAs (18:0, 18:2n-6, 22:1n-9). The others 20 FAs had all together a percentage sum < 5%. The lipid profile that we found in the muscle of *T. bernacchii* is in agreement with results available in the literature for the same teleost (Phleger et al, 1999; Wurzberg et al 2011) and for other species of the same genus, such as *Trematomus newnesi* (Lund and Sidell, 1992) and *Trematomus lepidorhinus* (Hagen et al. 2000). In general, all authors remarked that 14:0, 16:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-9, 20:5n-3 and 22:6n-3 were the main FAs, in agreement with our data.

On the average, SFA, MUFAs and PUFAs contributed 19.6±0.2%, 54.4±0.1% and 26.0±0.2% of total FAs, respectively. The high percentage of unsaturation (PUFA + MUFA, ~80%) is a response to low water temperature (-1.8°C): unsaturated fatty acids enhance membrane fluidity associated with cold tolerance in fish (Pörtner et al, 2007, Fujisawa et al, 2010). Similar percentual unsaturation was reported in literature for the same species (Phleger et al, 1999) and in other notothenioid fishes living in cold waters (Kamler et al, 2001; Magãlhaes et al, 2010; Stowasser et al, 2012).

It is known that fatty acid composition of the diet influences, although partially, fish fatty acid profile (Peng et al. 2009). Comparing FAs profile of muscle of *T. bernacchii* with those of muscle of teleosts of the same family, *Notothenia rossii*, and *Notothenia coriiceps*, living in the same Antarctic habitat, we noted that *T. bernacchii* shows an FA composition more similar to the FA profile of *N. rossii* than the FAs profile of *N. coriiceps* (Magalhães et al, 2010). This similarity is probably due to the same diet of *T. bernacchii* and *N. rossii*, that are voracious predators of smaller fishes, amphipods, polychaete and krill, whereas *N. coriiceps* is an omnivorous known to feed on a wide range of food, mainly salps, algae amphipods and isopods (Barrera-Oro, 2003, Wurzberg et al. 2011).

In terms of mass fraction of FAs in the muscle of *Trematomus bernacchii*, data reported in Table 5.4 are among the first ever published for a notothenioid fish, and it is therefore not possible to make any comparisons. Total FAs accounted for 46.8±0.2 mg g^{-1} dw, corresponding to 936±4 mg $100g^{-1}$ ww, i.e. ~0.94% ww. With respect to extracted total lipids, amounting to 1.1±0.2% ww (Table 5.1), FAs content was ~85%, in agreement with published data reporting that triacylglycerols (TAGs) represent up to 89% of total lipids in the muscle of *T. bernacchii* (Phleger et al. 1999). Consequently, our data indicated that the transesterification process is complete, giving derivatization of all FAs obtained from hydrolyzation of TAGs. Quantified FAs mass fraction were: SFA, 9.1±0.1 mg g^{-1} dw; MUFA, 25.5±0.1 mg g^{-1} dw; and PUFA, 12.2± 0.1 mg g^{-1} dw.

Characterizing biological factors associated with species-specific accumulation of contaminants or environmental stressful conditions is one of the major focuses in ecotoxicological and environmental chemical studies. The relationship between environmental pollution or stressful habitat conditions and the biological response of marine organisms based only on lipid and FAs biomarkers has seldom been investigated (Gladyshev et al. 2012; Perrat et al. 2013; Fokina et al. 2013; Fokina 2015; Gonçalves et al, 2016). This is probably due to the fact that each FAs was quantified as percentage *vs* total

FAs. In such measurements, FAs percent variation depends not only on external inputs but also on the percentage modification of others fatty acids and, in these conditions, it is more difficult to find cause/effect relationships. The identification of the absolute variation of any single fatty acid, measured in mass fraction, should be more useful to increase knowledge on the relationship between environmental stressful conditions and biological response of organisms. The method here proposed could be applied to any type of tissue, after a simple optimization of instrumental parameters. In particular, this work provides a valuable tool to better study the response of *T. bernacchii,* a key-specie in Antarctic environments, to stressful environmental conditions such as exposition to pollutants, providing a contribution for one of the eighty priority questions to be answered in the next two decades in Antarctic science, how next-generation contaminants will affect Antarctic and Southern Ocean biota.

5.5 Conclusions

A reliable and accurate absolute quantification of fatty acids, in terms of mass fraction, was performed in the muscle of *T. bernacchii*. To reach our goal we optimized and validated an analytical methodology based on microwave-assisted extraction of lipids from a lyophilized sample, derivatization of lipid extract using $NaOCH_3$, and FAMEs separation and identification by GC-MS. The lyophilization step before microwave-assisted extraction of lipids was introduced to increase the accuracy of lipid extraction and derivatization. The microwave-assisted lipid extraction allowed a minimum sample handling and a fast and reliable extraction at low temperatures that minimized fatty acids peroxidation. The optimization of the method required some modification of the temperature program of GC-analysis with respect to the one suggested from Agilent (David, 2003), to obtain optimal conditions for the best peak resolution and the minimum run time (43 min). This work could be useful to better study the response of *T. bernacchii,* a key-specie in Antarctic environment, to stressing environmental conditions. Giving the possibility to quantify each single FA as mass fraction, this work provides a valuable tool: i) to add useful information on the mechanism of bioaccumulation and biomagnification of contaminants in the trophic chains, and ii) to provide rapid and significant insights in the assessment of the biochemical and physiological status of marine organisms exposed to environmental stressful conditions.

5.6 Acknowledgements

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5.7 Figures, Tables and Supplementary materials

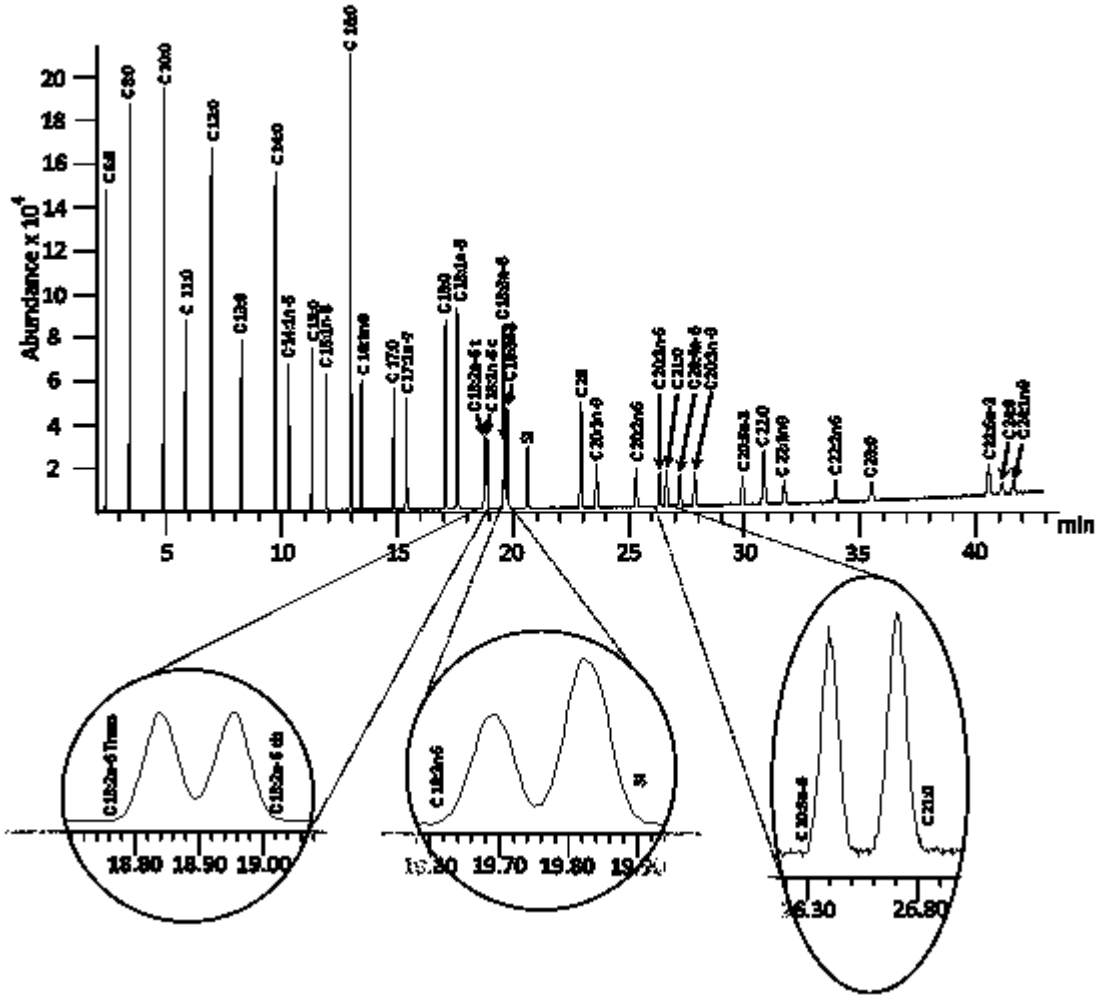

Figure. 5.1 Example of chromatogram of 37-Component FAME mix obtained with optimized instrumental parameters.

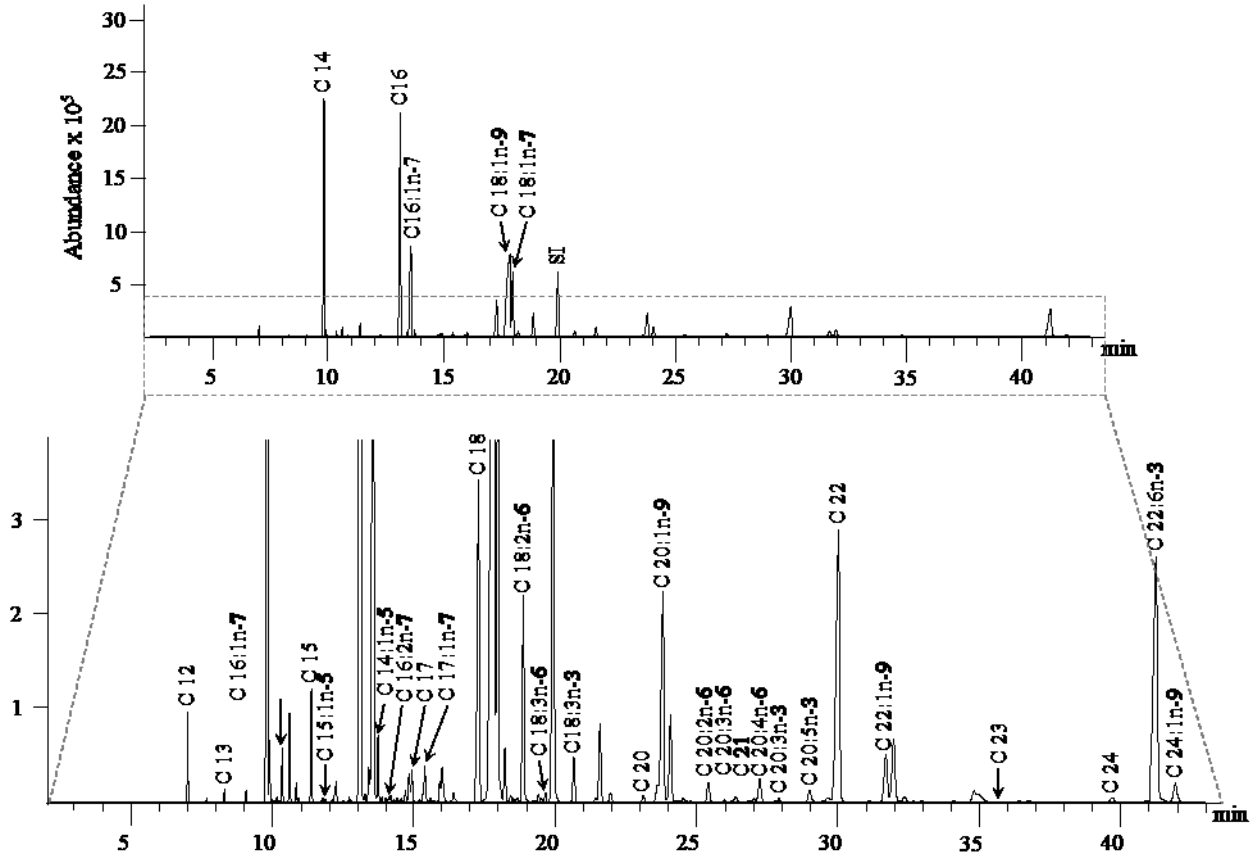

Figure 5.2. Representative chromatogram of total FAMEs from the muscle of *T. bernacchii*.

Table 5.1. Lipid percentage in muscle of *Trematomus bernacchii* and comparison with published data

Reference	Site, Antarctica	% FAs ww	% Fas dw	Extraction method
This work	Terra Nova Bay, Ross Sea	1.1 ± 0.2	5.8 ± 1.1	MAE
		1.3 ± 0.2	6.9 ± 1.2	Bligh and Dyer
Clarke et al. 1984	Southwest, Ross Sea	1.1 ± 0.4		Bligh and Dyer
Phleger et al. 1999	Mawson Station, East Antarctica		4.6 ± 2.7	Bligh and Dyer
Wurzberg et al. 2011	Weddell Sea		2.9 ± 1.4	Folch[a]
Corsolini et al. 2006	Terra Nova Bay, Ross Sea	0.86 ± 0.12		Soxhlet

[a]Folch et al., 1957

Table 5.2. Optimization of resolution between 20:3n-6/21:0, 19:0/18:3n-6, and *cis* and *trans* 18:2n-6.

Starting temperature/ (hold, min)	Temperature rate (°C/min)/ Final temperature (°C)(hold, min)	Temperature rate (°C/min)/ Final temperature (°C)/hold(min)	Total time	Rs 20:3n-6/21:0	Rs 19:0/18:3n-6	Rs 18:2n-6c/t
50/1	25/200	3/230(18)	35	0.39	UP	UP (S1)
50/1	25/200	1/230(18)	55	1.17	UP	UP
50/1	10/200	1/230(18)	64	1.09	0.41	UP
50/1	5/200	1/230(1)	62	1.38	0.97	0.88 (S2)
100/1	5/200(1)	1/230(1)	53	1.62	1.13	0.89 (S3)
100/1	25/150 5/200(1)	1/230(1)	43	1.5	0.87	0.80

UP =Unresolved Peaks
S1,S2,S3 indicate Figures in “Supplementary Material” reporting examples of chromatograms obtained with the corresponding conditions.

Table 5.3. Performance data of the optimized analytical methodology.

FA	t_r (min)	Linearity range, µg/ml (Std 1-Std 6)	r^2	Slope, *b*	Intercept	$S_{y/x}$	S_a	LOD µg/ml	LOQ µg/ml
12:0	7.031	48.16-215.8	0.9962	$14.5 \cdot 10^{-3}$	$138 \cdot 10^{-3}$	$59.6 \cdot 10^{-3}$	$55.5 \cdot 10^{-3}$	12.6	38.3
13:0	8.331	23.78-106.6	0.9968	$17.0 \cdot 10^{-3}$	$-16.7 \cdot 10^{-3}$	$31.2 \cdot 10^{-3}$	$29.1 \cdot 10^{-3}$	5.66	17.2
14:0	9.816	48.14-215.8	0.9978	$15.8 \cdot 10^{-3}$	$33.3 \cdot 10^{-3}$	$48.8 \cdot 10^{-3}$	$45.5 \cdot 10^{-3}$	9.51	28.8
14:1n-5	10.378	24.08-107.9	0.9982	$6.60 \cdot 10^{-3}$	$-10.7 \cdot 10^{-3}$	$9.18 \cdot 10^{-3}$	$8.57 \cdot 10^{-3}$	4.28	13.0
15:0	11.405	24.07-107.9	0.9980	$17.1 \cdot 10^{-3}$	$-31.8 \cdot 10^{-3}$	$25.5 \cdot 10^{-3}$	$23.8 \cdot 10^{-3}$	4.60	13.9
15:1n-5	12.008	23.86-107.0	0.9954	$7.19 \cdot 10^{-3}$	$-27.2 \cdot 10^{-3}$	$16.1 \cdot 10^{-3}$	$15.0 \cdot 10^{-3}$	6.89	20.9
16:0	13.110	72.22-323.7	0.9974	$14.8 \cdot 10^{-3}$	$95.7 \cdot 10^{-3}$	$74.6 \cdot 10^{-3}$	$69.6 \cdot 10^{-3}$	15.5	47.0
16:1n-7	13.533	24.08-107.9	0.9954	$7.17 \cdot 10^{-3}$	$-26.1 \cdot 10^{-3}$	$16.3 \cdot 10^{-3}$	$15.1 \cdot 10^{-3}$	6.97	21.1
17:0	14.972	24.00-107.6	0.9565	$11.6 \cdot 10^{-3}$	$124 \cdot 10^{-3}$	$82.3 \cdot 10^{-3}$	$76.7 \cdot 10^{-3}$	21.8	66.0
17:1n-7	15.500	23.72-107.9	0.9946	$7.24 \cdot 10^{-3}$	$-27.1 \cdot 10^{-3}$	$17.9 \cdot 10^{-3}$	$16.5 \cdot 10^{-3}$	7.53	22.8
18:0	17.263	48.14-215.8	0.9960	$15.5 \cdot 10^{-3}$	$-85.0 \cdot 10^{-3}$	$64.6 \cdot 10^{-3}$	$60.2 \cdot 10^{-3}$	12.9	39.9
18:1n-9	17.739	71.99-323.7	0.9966	$5.80 \cdot 10^{-3}$	$-64.6 \cdot 10^{-3}$	$34.2 \cdot 10^{-3}$	$32.0 \cdot 10^{-3}$	18.2	55.1
18:2n-6c	19.004	24.06-107.9	0.9928	$8.80 \cdot 10^{-3}$	$-58.2 \cdot 10^{-3}$	$25.1 \cdot 10^{-3}$	$23.4 \cdot 10^{-3}$	8.77	26.6
18:2n-6t	18.882	24.06-107.9	0.9888	$8.90 \cdot 10^{-3}$	$-58.2 \cdot 10^{-3}$	$25.4 \cdot 10^{-3}$	$23.6 \cdot 10^{-3}$	8.76	26.5
18:3n-6	19.694	24.07-107.9	0.9896	$3.61 \cdot 10^{-3}$	$29.3 \cdot 10^{-3}$	$15.6 \cdot 10^{-3}$	$14.5 \cdot 10^{-3}$	13.3	40.3
18:3n-3	20.703	24.70-107.9	0.9902	$4.79 \cdot 10^{-3}$	$24.2 \cdot 10^{-3}$	$15.8 \cdot 10^{-3}$	$14.8 \cdot 10^{-3}$	10.2	30.9
20:0	23.215	48.16-215.8	0.9948	$12.9 \cdot 10^{-3}$	$-27.1 \cdot 10^{-3}$	$62.7 \cdot 10^{-3}$	$58.4 \cdot 10^{-3}$	14.9	45.2
20:1n-9	23.824	24.08-107.6	0.9980	$5.39 \cdot 10^{-3}$	$-12.1 \cdot 10^{-3}$	$7.93 \cdot 10^{-3}$	$7.41 \cdot 10^{-3}$	4.53	13.7
20:2n-6	25.488	24.07-107.9	0.9958	$7.37 \cdot 10^{-3}$	$-26.7 \cdot 10^{-3}$	$16.0 \cdot 10^{-3}$	$14.9 \cdot 10^{-3}$	6.69	20.7
20:3n-6	26.469	24.05-107.9	0.9829	$7.35 \cdot 10^{-3}$	$-30.0 \cdot 10^{-3}$	$32.3 \cdot 10^{-3}$	$30.1 \cdot 10^{-3}$	13.5	41.0
21:0	26.933	23.95-107.9	0.9894	$12.1 \cdot 10^{-3}$	$-37.9 \cdot 10^{-3}$	$41.9 \cdot 10^{-3}$	$39.0 \cdot 10^{-3}$	10.6	32.1
20:4n-6	27.310	23.77-107.9	0.9886	$3.21 \cdot 10^{-3}$	$17.7 \cdot 10^{-3}$	$11.5 \cdot 10^{-3}$	$10.6 \cdot 10^{-3}$	10.9	33.1
20:3n-3	28.001	24.07-104.5	0.9950	$4.88 \cdot 10^{-3}$	$-15.3 \cdot 10^{-3}$	$11.0 \cdot 10^{-3}$	$10.0 \cdot 10^{-3}$	6.77	20.5
20:5n-3	29.978	24.40-107.9	0.9954	$3.86 \cdot 10^{-3}$	$-20.0 \cdot 10^{-3}$	$8.57 \cdot 10^{-3}$	$8.00 \cdot 10^{-3}$	6.84	20.8
22:0	31.229	48.06-215.3	0.9976	$11.2 \cdot 10^{-3}$	$0.61 \cdot 10^{-3}$	$35.7 \cdot 10^{-3}$	$33.3 \cdot 10^{-3}$	9.84	29.8
22:1n-9	32.030	24.07-107.9	0.9956	$5.00 \cdot 10^{-3}$	$-18.4 \cdot 10^{-3}$	$10.0 \cdot 10^{-3}$	$10.3 \cdot 10^{-3}$	6.78	20.5
23:0	35.906	24.07-107.6	0.9882	$10.7 \cdot 10^{-3}$	$-39.4 \cdot 10^{-3}$	$38.9 \cdot 10^{-3}$	$36.3 \cdot 10^{-3}$	11.2	33.9
24:0	40.987	48.14-215.8	0.9982	$9.36 \cdot 10^{-3}$	$-53.8 \cdot 10^{-3}$	$14.9 \cdot 10^{-3}$	$25.1 \cdot 10^{-3}$	8.83	26.8
22:6n-3	41.207	24.08-107.9	0.9892	$4.63 \cdot 10^{-3}$	$-36.1 \cdot 10^{-3}$	$16.1 \cdot 10^{-3}$	$15.0 \cdot 10^{-3}$	10.7	32.4
24:1n-9	42.048	24.07-107.9	0.9763	$4.25 \cdot 10^{-3}$	$-19.0 \cdot 10^{-3}$	$22.0 \cdot 10^{-3}$	$20.5 \cdot 10^{-3}$	16.0	48.4

$S_{y/x}$ = standard deviation of the residuals
S_a = standard deviation of the intercept of regression line

Table 5.4. Fatty acid composition (as % *vs* total Fas and as mg g^{-1} tissue dry weight) of muscle of *T. bernacchii*. Data are reported as mean±SD of nine organisms.

Fatty Acid	% Total Fas	mg g^{-1} dw
12:0	0.12±0.1	0.054±0.018
13:0	0.03±0.1	0.015±0.01
14:0	8.38±0.9	3.89±0.05
14:1n-5	0.40±0.1	0.187±0.05
15:0	0.29±0.1	0.136±0.01
15:1n-5	0.01±0.001	0.003±0.001
16:0	9.10±0.8	4.24±0.5
16:1n-7	11.5±1.2	5.37±0.7
16:2n-7	0.08±0.03	0.038±0.02
17:0	0.10±0.03	0.045±0.006
17:1n-7	0.43±0.1	0.203±0.04
18:0	1.36±0.3	0.636±0.2
18:1n-9	26.7±0.9	12.54±0.4
18:1n-7	6.52±0.3	3.05±0.2
18:2n-6c	2.45±0.4	1.15±0.02
18:3n-6	0.19±0.1	0.088±0.05
18:3n-3	0.83±0.2	0.391±0.07
20:0	0.05±0.01	0.025±0.01
20:1n-9	5.59±0.5	2.63±0.2
20:2n-6	0.31±0.1	0.148±0.07
20:3n-6	0.27±0.2	0.125±0.01
21:0	0.03±0.01	0.014±0.01
20:4n-6	0.58±0.2	0.374±0.01
20:3n-3	0.09±0.01	0.043±0.03
20:5n-3	10.3±0.4	4.82±0.4
22:0	0.09±0.01	0.041±0.007
22:1n-9	2.19±0.4	1.04±0.2
23:0	0.02±0.01	0.010±0.01
24:0	0.07±0.01	0.032±0.02
22:6n-3	10.9±1.3	5.14±0.8
24:1n-9	0.99±0.2	0.469±0.01
Total	100.0±0.3	46.85±0.16

Table S5.1. Percentage of lipids extracted from muscle of *Trematomus bernacchii*.

N° sample	% lipid ww		
	MAE + 46yophilisation step	MAE	Bligh & Dyer
1	0.9±0.3	0.2±0.2	1.4 ± 0.3
2	1.0±0.1	0.3±0.1	1.1 ± 0.1
3	0.9±0.2	0.4±0.2	1.3 ± 0.2
4	1.3±0.2	0.3±0.2	1.5 ± 0.2
5	1.1±0.1	0.2±0.1	1.2 ± 0.1
6	0.9±0.1	0.3±0.1	1.3 ± 0.1
7	1.3±0.3	0.3±0.1	1.5 ± 0.3
8	1.2±0.3	0.2±0.2	1.2 ± 0.1
9	1.2±0.1	0.4±0.1	1.0 ± 0.2
Mean±SD	1.1±0.2	0.3±0.1	1.3 ± 0.2

Table S5.2. Nomenclature of fatty acids cited in the text.

FA	IUPAC name	Other names
12:0	Dodecanoic acid	Lauric acid
13:0	Tridecanoic acid	Tridecylic acid
14:0	Tetradecanoic acid	Myristic acid
14:1n-5	(9Z)-Tetradec-9-enoic acid	Myristoleic acid
15:0	Pentadecanoic acid	
15:1n-5	(10Z)-Pentadec-10-enoic acid	Cis-10-Pentadecenoic acid
16:0	Hexadecanoic acid	Palmitic acid
16:1n-7	(9Z)-Hexadec-9-enoic acid	Palmitoleic acid
17:0	Heptadecanoic acid	Margaric acid
17:1n-7	(10Z)-Heptadec-10-enoic acid	Cis-10-heptadecenoic acid
18:0	Octadecanoic acid	Stearic acid
18:1n-9	(9Z)-Octadec-9-enoic acid	Oleic acid
18:2n-6c	(9Z,12Z)-9,12-Octadecadienoic acid	Linoleic acid
18:2n-6t	(9E,12E)-Octadeca-9,12)-dienoic acid	Linolelaidic acid
18:3n-6	(6Z,9Z,12Z)-Octadeca-6,9,12-trienoic acid	γ-Linolenic acid
18:3n-3	(9Z,12Z,15Z)-Octadeca-9,12,15-trienoic acid	α-Linolenic acid
20:0	Eicosanoic acid	Arachic acid
20:1n-9	(11Z)-Icosa-11-enoic acid	Gondoic acid
20:2n-6	(11Z,14Z)-Icosa-11,14-dienoic acid	Eicosadienoic acid
20:3n-6	(8Z,11Z,14Z)-Icosa-8,11,14-trienoic acid	Dihomo-γ-linolenic acid
21:0	Heneicosanoic acid	Henicosanoic acid
20:4n-6	(5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoic acid	Arachidonic acid
20:3n-3	(11Z,14Z,17Z)-Icosa-11,14,17-trienoic acid	Eicosatrienoic acid
20:5n-3	(5Z,8Z,11Z,14Z,17Z)-Icosa-5,8,11,14,17-pentaenoic acid	Eicosapentaenoic acid
22:0	Docosanoic acid	Behenic acid
22:1n-9	(13Z)-Docos-13-enoic acid	Erucic acid.
23:0	Tricosanoic acid	Tricosylic acid
24:0	Tetracosanoic acid	Lignoceric acid
22:6n-3	(4Z,7Z,10Z,13Z,16Z,19Z)-Docosa-4,7,10,13,16,19-hexaenoic acid	Docosahexaenoic acid
24:1n-9	(15Z)-Tetracos-15-enoic acid	Nervonic acid

Table S5.3. Recovery test for Fas present in the muscle of *Trematomus bernacchii*.

FA	Recovery %	
	Std1	Std6
12:0	88±2	92±3
13:0	91±3	86±2
14:0	87±2	94±2
14:1n-5	89±4	88±3
15:0	81±2	86±3
15:1n-5	101±4	94±2
16:0	100±3	99±2
16:1n-7	98±2	99±3
17:0	86±2	93±3
17:1n-7	93±3	91±3
18:0	94±2	102±4
18:1n-9c	99±3	103±3
18:2n-6c	105±4	111±4
18:3n-6	100±3	102±5
18:3n-3	88±3	98±2
20:0	102±4	94±3
20:1n-9	99±5	98±3
20:2n-6	101±4	100±4
20:3n-6	105±3	101±5
21:0	105±5	97±2
20:4n-6	93±2	102±4
20:3n-3	105±3	99±3
20:5n-3	100±4	101±2
22:0	96±3	95±3
22:1n-9	101±2	86±2
23:0	95±3	97±3
24:0	81±2	81±2
22:6n-3	101±2	105±3
24:1n-9	94±3	89±3

Table S5.4. Intra-day precision. Data are expressed in mg g^{-1} dw.

FA	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Media	DS	DS%
12	0.057	0.052	0.055	0.048	0.062	0.045	0.055	0.006	11
13	0.012	0.015	0.019	0.014	0.017	0.016	0.016	0.002	16
14	3.39	3.23	3.12	3.14	3.12	3.15	3.25	0.106	3
14:1n-5	0.187	0.170	0.166	0.164	0.151	0.152	0.172	0.013	8
15	0.136	0.138	0.120	0.119	0.140	0.135	0.135	0.009	7
15:1n-5	0.004	0.003	0.003	0.004	0.003	0.003	0.003	0.001	20
16	4.24	4.02	3.93	3.93	3.91	3.94	4.08	0.126	3
16:1n-7	5.37	5.07	4.91	4.97	4.93	4.97	5.13	0.172	3
16:2n-7	0.038	0.035	0.031	0.032	0.033	0.030	0.035	0.003	8
17	0.041	0.042	0.049	0.051	0.052	0.043	0.048	0.005	10
17:1n-7	0.203	0.168	0.161	0.165	0.162	0.165	0.186	0.016	9
18	0.645	0.615	0.609	0.629	0.600	0.621	0.620	0.002	3
18:1n-9	12.5	12.1	11.9	12.1	12.0	12.1	12.2	0.217	2
18:1n-7	3.08	2.97	2.91	2.94	2.93	2.96	3.00	0.060	2
18:2n-6	1.15	1.11	1.09	1.10	1.09	1.09	1.12	0.024	2
18:3n-6	0.088	0.078	0.080	0.086	0.093	0.091	0.089	0.006	7
18:3n-3	0.355	0.372	0.366	0.399	0.383	0.397	0.384	0.018	5
20	0.019	0.023	0.029	0.023	0.027	0.024	0.026	0.003	14
20:1n-9	2.63	2.57	2.42	2.41	2.59	2.59	2.60	0.094	4
20:2n-6	0.148	0.135	0.132	0.157	0.141	0.133	0.148	0.010	7
20:3n-6	0.125	0.133	0.117	0.129	0.127	0.139	0.130	0.007	6
21	0.014	0.011	0.017	0.013	0.014	0.015	0.014	0.002	14
20:4n-6	0.374	0.354	0.340	0.365	0.389	0.333	0.369	0.021	6
20:3n-3	0.043	0.047	0.044	0.042	0.051	0.045	0.047	0.003	7
20:5n-3	4.82	4.86	4.84	4.91	4.88	4.82	4.88	0.038	1
22	0.037	0.044	0.046	0.049	0.043	0.040	0.044	0.004	10
22:1n-9	1.04	1.07	0.973	1.05	1.03	0.98	1.05	0.038	4
23	0.010	0.014	0.017	0.013	0.013	0.014	0.014	0.002	16
24	0.027	0.031	0.030	0.027	0.033	0.031	0.029	0.003	11
22:6n-3	5.14	5.11	4.92	4.78	4.96	4.71	5.03	0.172	3
24:1n-9	0.433	0.475	0.440	0.492	0.436	0.501	0.480	0.030	6

Table S5.5. Inter-day precision. Data are expressed in mg g^{-1} dw.

FA	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Media	DS	DS%
12	0.044	0.052	0.055	0.060	0.049	0.041	0.052	0.007	14
13	0.015	0.010	0.017	0.014	0.013	0.012	0.014	0.002	18
14	3.45	3.29	3.58	3.34	3.22	3.14	3.34	0.16	5
14:1n-5	0.187	0.167	0.197	0.179	0.160	0.216	0.192	0.020	11
15	0.140	0.132	0.120	0.137	0.149	0.118	0.137	0.012	9
15:1n-5	0.003	0.002	0.003	0.004	0.002	0.002	0.003	0.001	25
16	4.24	4.12	4.03	4.42	4.15	4.58	4.35	0.21	5
16:1n-7	5.37	5.06	4.49	5.34	5.63	5.58	5.42	0.26	5
16:2n-7	0.038	0.033	0.047	0.040	0.033	0.036	0.039	0.005	13
17	0.045	0.043	0.50	0.045	0.034	0.049	0.046	0.006	12
17:1n-7	0.203	0.175	0.222	0.176	0.183	0.230	0.215	0.024	11
18	0.636	0.644	0.660	0.611	0.607	0.678	0.653	0.028	4
18:1n-9	12.5	12.8	12.5	11.9	12.1	13.1	12.6	0.443	4
18:1n-7	3.08	3.25	3.10	2.91	3.07	3.17	3.13	0.114	4
18:2n-6	1.15	1.19	1.13	1.06	1.12	1.15	1.15	0.043	4
18:3n-6	0.088	0.072	0.079	0.095	0.069	0.087	0.085	0.010	12
18:3n-3	0.411	0.352	0.411	0.423	0.379	0.359	0.395	0.030	8
20	0.025	0.022	0.026	0.031	0.019	0.030	0.027	0.005	17
20:1n-9	2.88	2.51	2.47	2.64	2.75	2.87	2.76	0.176	6
20:2n-6	0.148	0.133	0.121	0.131	0.149	0.117	0.141	0.013	9
20:3n-6	0.125	0.113	0.133	0.101	0.110	0.119	0.119	0.011	9
21	0.014	0.018	0.011	0.017	0.020	0.016	0.016	0.003	19
20:4n-6	0.321	0.370	0.342	0.362	0.389	0.398	0.374	0.029	8
20:3n-3	0.043	0.048	0.047	0.042	0.037	0.040	0.045	0.004	9
20:5n-3	4.82	4.71	4.55	4.59	4.31	4.56	4.62	0.172	4
22	0.041	0.050	0.038	0.048	0.046	0.033	0.043	0.007	15
22:1n-9	1.01	1.13	1.10	1.06	1.03	1.19	1.11	0.067	6
23	0.010	0.017	0.018	0.016	0.021	0.021	0.018	0.004	22
24	0.030	0.025	0.030	0.038	0.033	0.031	0.031	0.004	14
22:6n-3	5.14	4.79	5.31	4.90	4.86	5.52	5.19	0.288	6
24:1n-9	0.474	0.435	0.428	0.418	0.375	0.457	0.449	0.034	8

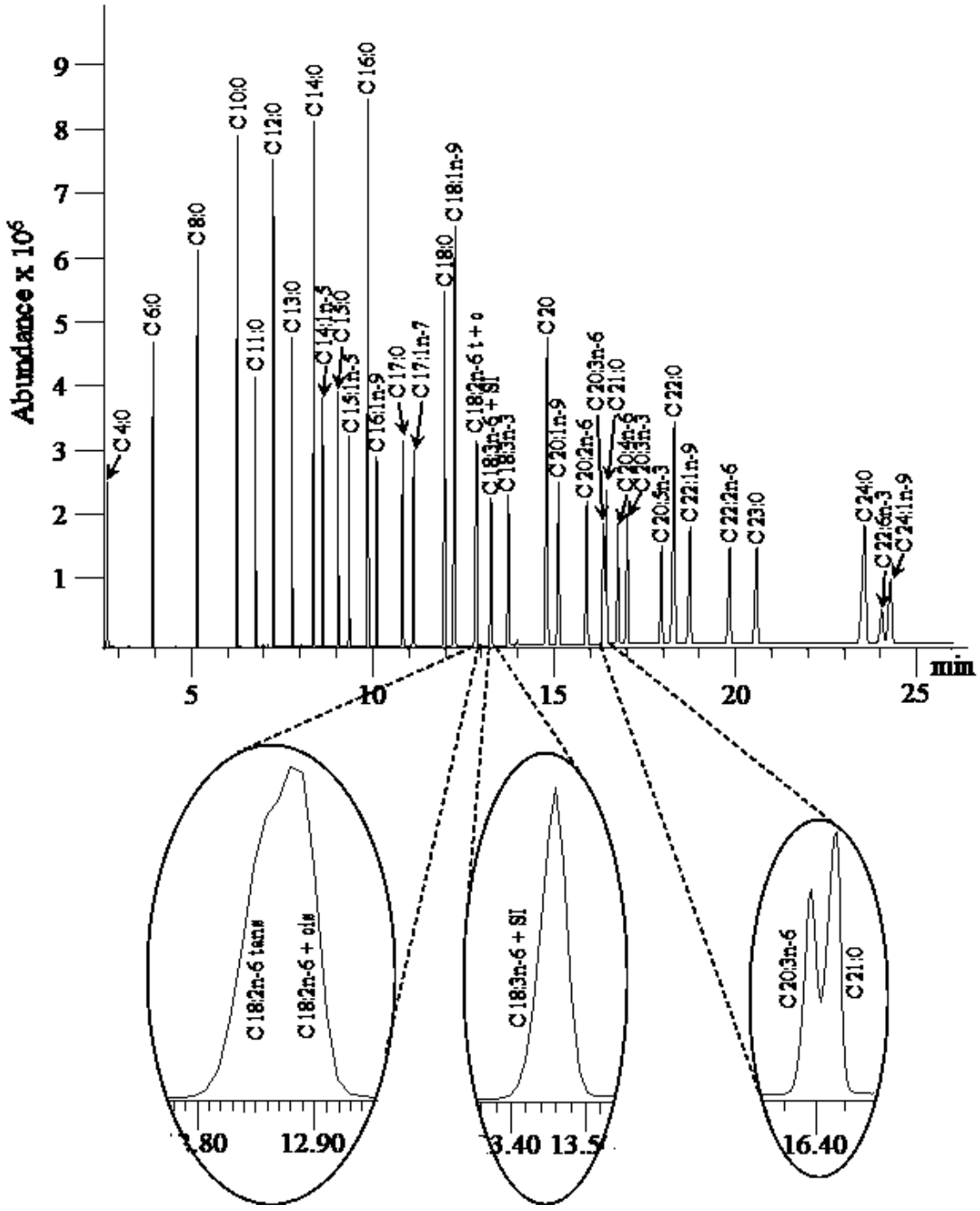

Figure S5.1. Example of chromatogram of 37-Component FAME mix obtained with instrumental parameters from Application Note n. 5988-5871 EN, Agilent (David, 2003).

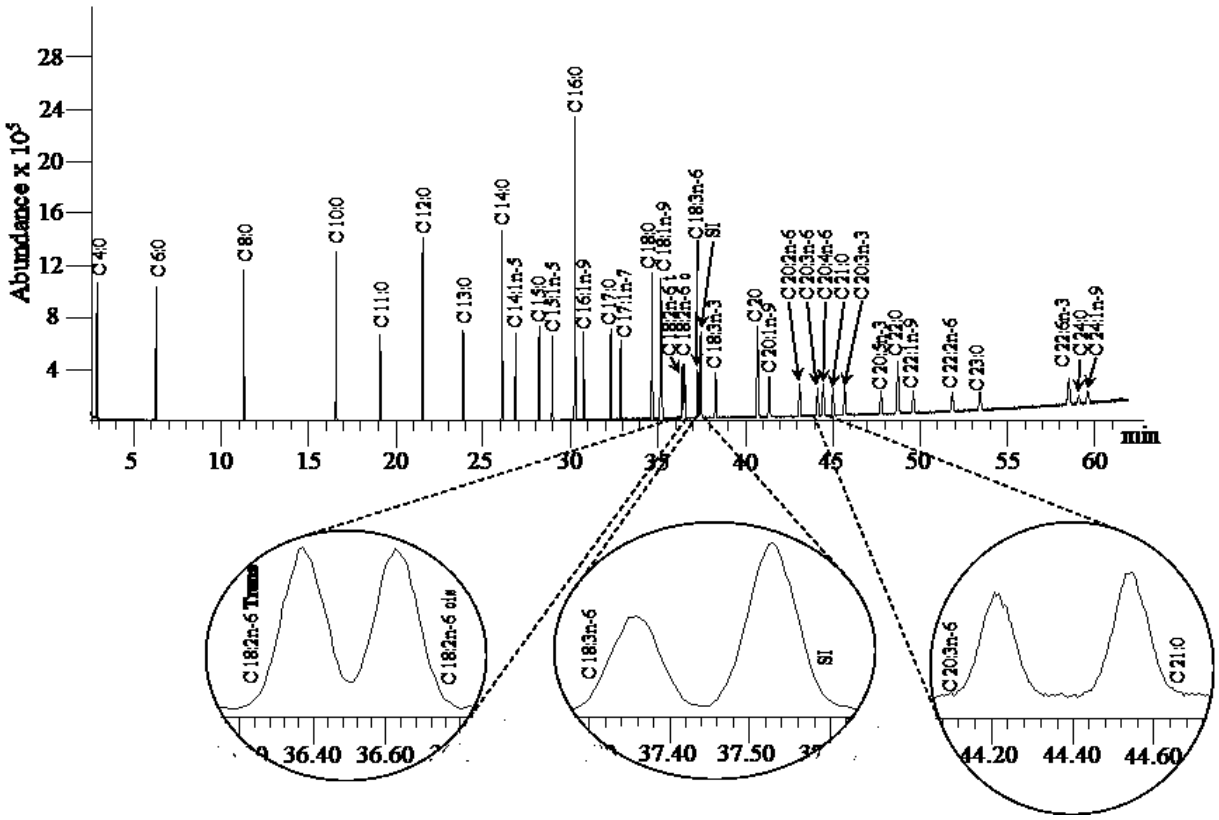

Figure S5.2. Example of chromatogram of 37-Component FAME mix obtained with program temperature as in Table 5.2.

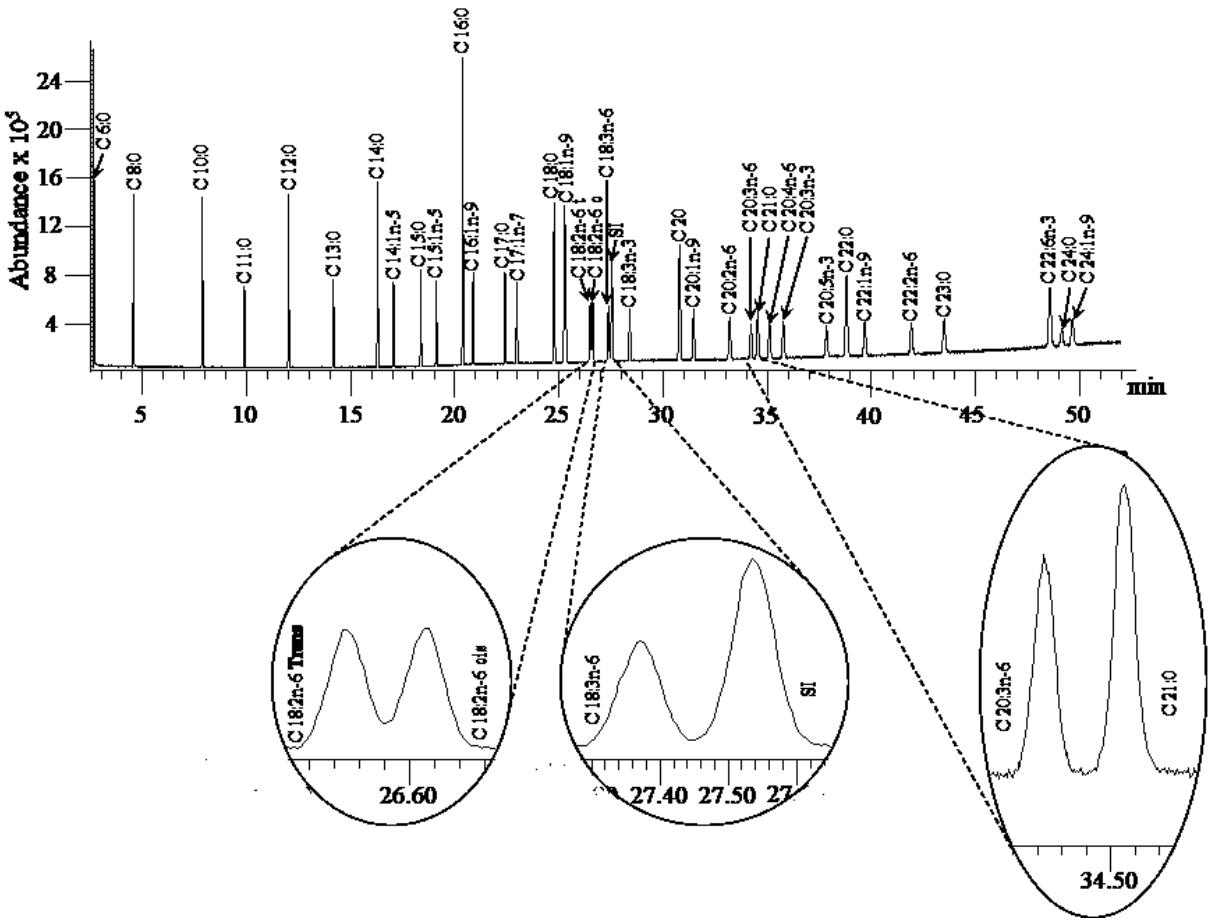

Figure S5.3. Example of chromatogram of 37-Component FAME mix obtained with program temperature as in Table 5.2.

5.8 References

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6 Heat shock influences the fatty acid composition of the muscle of the Antarctic fish *Trematomus bernacchii*

6.1 Abstract

In the Ross Sea region (average temperature of -1.87 °C), shelf water warming up to +0.8-+1.4 °C is predicted by 2200, so there is an urgent need to understand how organisms can respond to rising temperatures. In this study, we analyzed the effect of a heat shock on the fatty acid (FAs) composition of muscle of the Antarctic teleost *Trematomus bernacchii*, caught in Terra Nova Bay (Ross Sea), and held in fish tanks at 0, +1 or +2 °C, for 1, 5 and 10 days. In general, heat shock produced, beyond that a reduction in total lipid content correlated to the temperature, an increase in the percentage of saturated FAs, and a decrease in mono-unsaturated FAs; however, the level of poly-unsaturated FAs did not seem to directly correlate with temperature. Principal component analysis helped us to hypothesize that both temperature and exposure time affect the composition of FAs in the muscle mainly through an alteration of the methabolic patways of FAs. In this study we demonstrated that *T. bernacchii* was capable to rapidly acclimatize to a heat shock. This study contributes to increasing knowledge on the effect of temperature on the lipid composition of *T. bernachii* and are complementary to previous studies on the gene expression and biochemistry of this species face to multiple stress.

6.2 Introduction

Global climate change is causing an increase in mean world temperature, fluctuations and frequency of temperature extremes. The recent models of global climate change by IPCC (2014) predict an increase in the global mean surface temperature of 3-6 °C by the year 2100. In the past, temperature changes have caused modifications in species distribution, abundance and ecosystems (Falfushynska et al. 2014). Recent climate changes have led to a number of documented alterations in ecosystems throughout the world, directly affecting the metabolism of living organisms and, indirectly, the dynamics of

trophic chains (Brodersen et al. 2011; Paital and Chainy, 2014, Pörtner et al. 2007). Rising temperatures in general influence the physiological and biogeochemical processes of organisms, not least the composition of lipid tissue and cellular membrane fluidity in phytoplankton, zooplankton and fishes (Hixson et al. 2016, Constable et al. 2014). What allows different species to survive to climate change is their ability to adapt (Falfushynska et al. 2014) or colonize new territories with appropriate environmental conditions.

Antarctica is a pristine environment that, although far from the anthropic environment, is undergoing the consequences of global change, both in terms of increasing anthropogenic contamination (Illuminati et al. 2015; Barbaro et al. 2016; Illuminati et al. 2017; Truzzi et al. 2017a), and in terms of temperature increase (Constable et al. 2014). In the Ross Sea region, shelf water warming up to 0.8-1.4 °C is predicted by 2200 (Timmermann and Helmmer, 2013). This change is leading to macroscopic consequences, such as melting ice, but far more subduedly, it is leading to significant changes in organisms that have adapted to live in such extreme conditions. Antarctic organisms live permanently at low temperatures (about -1.8 °C) and some species are considered highly stenothermal, i.e. are highly specialized to survive at low temperatures (Eastman, 1990; Colella et al. 2000; Pörtner et al. 2007; Palmerini et al. 2009; Machado et al. 2014), and generally they lose their ability to resist high temperatures of about +4 °C (Seebacher et al. 2005, Pörtner et al. 2007). Recent studies conducted in various groups of Antarctic notothenioid fishes demonstrated that some species are more tolerant to warmer temperatures due to climate change, and that they did not lose the capacity to respond to environmental changes (Seebacher et al. 2005; Podrabsky and Somero, 2006; Pörtner et al. 2007). Moreoever, the acclimation capacity of Antarctic fish (and other organisms) for thermal tolerance adjustment is species-specific (Enzor et al. 2013; Strobel et al. 2013). In ectothermic fishes, metabolic rate is strongly related to environmental temperature (Pernet et al. 2007; Machado et al. 2014; Sandersfeld et al. 2015).

Polar fishes are characterized by a high lipid content in tissues that cover important physiological roles (Sidell and Hazel 2002; Machado et al. 2014). Lipid metabolism is a dynamic system which includes endogenous metabolism and dietary intake of lipids, their accumulation in the form of triacylglycerols (TAG) and their catabolism (Sidell and Hazel 2002). Polar fishes have a high density of lipid droplets and of mitochondria (Sidell, 1998; Sidell and Hazel 2002; Grim et al. 2010); these characteristics indicate that polar fishes mainly have aerobic metabolism fueled mostly by lipid catabolism (Colella et al. 2000; Sidell

and Hazel 2002). Moreover, polar fishes are characterized by an high content of unsaturated fatty acids in the membrane cell to maintain the proper fluidity over a certain range in a so cold environment (Sinensky, 1974; Cossins, 1994). The response to a temperature change, i.e. homeoviscous adaptation, is essential for achieving the right fluidity necessary for the functionality of biological membranes (Roche and Pérès, 1984; Acierno et al. 1996).

In Antarctica, the notothenioid teleost *Trematomus bernacchii* (Boulenger, 1902), is considered a key species: it represents more than 90% of the abundance and biomass of coastal fish fauna at Terra Nova Bay (Vacchi et al. 1996), it is widely distributed and commonly found within the first 200 m of depth, and its ecology and reproductive biology have been well characterized (Gon and Heemstra, 1990). Due to these characteristics, it is often used as a reliable bio-indicator for environmental studies (Bargagli et al. 1998; Regoli et al. 2005; Corsolini et al. 2006; Borghesi et al. 2008; Illuminati et al. 2010; Ghosh et al. 2013; Cincinelli et al. 2016), therefore we chose *T. bernacchi* for the study here proposed.

Given the importance of lipids, the primary substrate of aerobic metabolism in Antarctic fishes, beyond that essential structural components of cells, we decided to test if *Trematomus bernacchii* is able to rapidly acclimatize to rising temperatures, by studying the fatty acids composition of muscle tissue of the fish exposed to heat shock in fish tanks. After more than 50 years of research focused on the adaptation of notothenioids to climate warming, there are still many questions that remain to be answered (Beers and Jayasundara 2015). Therefore this study may help gather important information on the acclimation of Antarctic fishes to rising temperatures, and can be complementary to previous studies on the gene expression and biochemistry of this species face to multiple stress.

6.3 Material and methods

6.3.1 Chemicals

All solvents and reagents were of HPLC grade: *n*-heptane and methanol (Baker, Philipsburg, NJ, USA); acetone, petroleum ether 35-60 °C, and anydrous sodium sulphate (Carlo Erba, Milano, Italy); sodium hydrogen sulfate anhydrous, extra pure (Scharlau, Sentmenat, Spain); sodium methoxide for synthesis (≥ 97%, Merck, Hohenbrunn, Germany); methyl ester of nonadecanoic acid used as internal standard (99.6%, Dr.

Ehrenstorfer GmbH, Germany); 37-component FAME mix (≥ 99%, Supelco, Bellefonte, PA, USA).

6.3.2 Sample collection and experimental design

During the 28th Italian Antarctic Expedition (austral summer 2014–2015), 66 sexually mature specimens of *T. bernacchii* (weight 136-333 g, length 22-30 cm) were caught by a fishing rod in Tethys Bay (74°42'052'' S, 164°02'267'' E) at the depth of ~30 m, very close to the ‘‘Mario Zucchelli’’ Station at Terra Nova Bay, Ross Sea, Northern Victoria Land. Six specimens were sacrificed just after capture, and used as “control group” (C0). The other fishes were rapidly held in a flow-through seawater fish tank at ambient temperature (-1.8 °C, controlled to within 0.1 °C) for a period of acclimation of 10 days. After the acclimation period, fishes were held in fish tanks at different temperatures: six fishes were mantained at -1.8 °C and sacrificed at 10 days, and used as “control group after acclimation” (C1). The other fishes were directly transferred in closed circuit tanks at 0, +1 or +2 °C (controlled to within 0.1 °C), where air was continuously infused, and sacrificed at 1, 5 and 10 days. For each treated-group, 6 specimens were used. During both the acclimation and the experiment period, fishes were fed with chopped fish muscle *ad libitum* (no substantial differences between groups in the amount of fish eaten were evidenced). The choice of these temperatures as thermal stress is based on shelf water warming until +0.8-+1.4 °C predicted by 2200 for the Ross Sea region (Timmermann and Helmmer, 2013). A short acclimation period was chosen because our aim was to investigate the ability of *T. bernacchii* to rapidly respond to rising temperatures. Sacrificed fishes were measured, weighed, and muscle tissue, such as other tissues, were isolated, frozen in liquid nitrogen, stored at -80 °C, and transported to Italy for fatty acid analysis.

6.3.3 Lipid extraction and fatty acid analysis

Fish fillets were minced, homogenized (homogenizer MZ 4110, DCG Eltronic), and divided into aliquots of about 1 g. Analyses were carried out on three aliquots *per* fish. Lipid extraction was carried out on lyophilized samples (Edwards EF4, Crawley, Sussex, England) by Microwave-Assisted Extraction (MAE) according to Truzzi et al (2017b). The extracts

were evaporated under laminar flow inert gas (N_2) until constant weight. After drying, the mass of extracted lipids was determined.

Fatty acid methyl esters (FAMEs) were prepared according to Canonico et al. (2016), using the methyl ester of nonadecanoic acid (19:0) as internal standard. FAMEs were determined on an Agilent-6890 GC System coupled to an Agilent-5973N quadrupole Mass Selective Detector (MSD). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAMEs. Instrumental conditions were as reported in Truzzi et al. (2017b): sample injections of 1 μL were made in a split mode ratio (1:5 or 1:10) using a glass cup liner (Agilent Liner, slittess, double taper 5583-4705). The inlet temperature was kept at 250 °C. Helium carrier gas (99.9999%, SOL, Italy) (8.0 psi) was used at a flow rate of 1 mL min^{-1}. The oven temperature started at 100 °C for 1 min, and it was subsequently increased to 150 °C at the rate of 25 °C min^{-1}, to 200 °C at the rate of 5 °C min^{-1} and to 230 °C at the rate of 1 °C min^{-1}, for a total run time of 43 min. Data collection, identification and quantification of FAs were as reported by Truzzi et al. (2017b). Retention time and mass spectra of 37-component FAME Mix standard were used to confirm the NIST identification of FAs in the sample. For each sample, at least three runs were performed on the GC-MS. The estimated limits of detection (LOD) and limits of quantification (LOQ), calculated as reported by Truzzi et al. (2014a, 2014b), ranged for each FAME from ~4 μg mL^{-1} to ~22 μg mL^{-1}, and from ~13 μg mL^{-1} to ~66 μg mL^{-1} (Truzzi et al. 2017b).

6.3.4 Statistical analysis

To compare total lipid percentage and FAs composition between groups, the one-way-ANOVA test, followed by the Multiple Range Test (Daniel, 2013) was performed after testing the homogeneity of variance with Levene's test. Significant differences were evaluated at the 95% confidence level. Principal Component Analysis (PCA) was carried out on standardized data; significant components were obtained through the Wold cross validation procedure (Wold, 1978). ANOVA test and PCA were performed using Statgraphics Plus 5.1 (2009, Manugistics Inc., Rockville, Maryland, USA).

6.4 Results

6.4.1 Lipid content

The percentage of dry weight (dw) in the muscle of *T. bernacchii* varied from 19% to 22% (mean 20%±1%), and no statistically significant differences were recorded between specimens of different groups. Total lipid percentages (%TL) *vs* dw of muscle of specimens of *T. bernacchii* subjected to different temperatures are reported in Fig. 6.1. Differences in total lipids percentage of the two control groups (fishes as captured, C0, and after acclimation, C1) were not statistically significant, as well as differences between acclimatized control group C1 and specimens exposed at 0 °C, for any length of time, and at 1 °C for 1 and 5 days. Specimens exposed at 1 °C for 10 days and at 2 °C for 1, 5 and 10 days showed a %TL statistically lower than control groups at any time, with a decreasing trend for animals exposed at 2 °C with increasing the exposure time. Morover, specimens exposed at 2 °C showed a %TL statistically lower than animals exposed at 0 °C.

6.4.2 FAs composition

Table 6.1 shows FAs composition of muscle of specimens of *T. bernacchii* from different groups. Only FAs >0.5% are reported, and the complete dataset is reported in Table S6.1 (see Supporting Information). Minor FAs, with a percentage <1% were excluded from any statistical analyses because their concentration were close to LOD. Fig. S6.1 (see Supporting Information) visualizes statistically significant results. Data on FAs composition of muscle did not show significant changes on lipid profile between control group C0 and acclimatized control group C1. Conversely, statistically significant differences were found in FAs composition between temperature-treated fishes and control groups. Concerning the most important saturated fatty acids (SFAs), myristic acid (14:0) showed a significant decrease after both 1 and 10 days of treatment with respect to control groups, for all tested temperatures. Palmitic (16:0) and stearic (18:0) acids increased significantly in all treated-groups, except for specimens exposed at 0 °C for 5 days, and at 2 °C for 10 days. Therefore, total SFAs are generally higher in temperature-treated specimens than in control ones, except for animals exposed at 0 °C for 1 day and 2 °C for 10 days. The most important mono-unsaturated fatty acids (MUFAs) showed in general a significant percentage reduction

in treated-groups *vs* control ones: palmitoleic acid (16:1n-7), significantly reduced only at 1 °C after 1 day, whereas oleic acid 18:1n-9 significantly decreased at all exposure times for 0 and 1 °C, and after 1 day of treatment for specimens exposed at 2 °C. *Cis*-vaccenic acid (18:1n-7) showed a significant reduction after 1 and 10 days at 0 °C, for all times of exposure at 1°C, and after 5 days at 2 °C. Gondoic acid (20:1n-9) showed a significant percentage reduction after 1 day at 1 and 2 °C, and after 10 days at 2 °C, whereas no variations were recorded at 0 °C with respect to control groups. Erucic acid (22:1n-9) showed a significant decrease only after 1 day for 2 °C. Total MUFAs showed in general a significant reduction with respect to control groups for all tested temperatures and times of exposure, except for specimens exposed at 0 °C for 5 days and at 2 °C for 10 days. The lowest total MUFAs percentages were recorded after 1 day for 0 and 1 °C. The essential fatty acid (EFA) eicosapentaenoic acid (EPA, 20:5n-3) showed values significantly higher than the control groups for all treated-groups. The highest values were recorded after 1 and 10 days at 0 and 1 °C. The other important EFA, docosaesaenoic acid (DHA, 22:6n-3) showed a different behavior for different temperatures and days of exposure. In particular, a significant increase was recorded in specimens exposed at 0 °C after 1 and 10 days, and in specimens exposed at 1 °C after 1 and 5 days. Conversely, a significant decrease was recorded in specimens exposed at 0 °C after 5 days, and in specimens exposed at 2 °C for all tested exposure times. Total omega3 poly-unsaturated fatty acids (PUFAs) showed a statistically significant increase after 1 day of exposure for all tested temperatures, that persisted until 5 days for specimens exposed at 0 °C, and until 10 days for those exposed at 1 °C. Linoleic acid (18:2n-6) did not show significant variations between groups. Overall, PUFAs (represented for about 80% by the sum of EPA and DHA) showed a significant increase after 1 day for all tested temperatures, with 0 and 1 °C producing the major increase. Values significantly higher than in control groups persisted after 5 days only for 1 °C, and after 10 days for 0 °C and 1 °C.

To better understand the relationships between FAs composition and exposure to different temperatures for different times, a multivariate analysis (Principal Component Analysis, PCA) was performed to reduce the dimensionality of the data set to few components that summarize the information contained in the overall data set. The percentage of total lipids (*vs* dw, %TL) were included in the data-set, together with FAs >1%. Applying principal component analysis to the data set (11 observations, 12 variables), it was possible to extract three significant, cross-validated principal components, that accounted for 86.5 % of the variability in the original data (Table 6.2).

On examining the loading matrix (Table 6.2) and the graphical distribution of analyzed groups on the reported biplots of PC1 *vs* PC2 (Fig. 6.2a), and PC1 *vs* PC3 (Fig. 6.2b), specimens were divided on the basis of their FAs composition. The first component separated control groups C0 and C1, and specimens exposed at 0 °C for 5 days (positive scores), characterized by higher percentages of 14:0 18:1n-7 and 18:1n-9, and lower percentages of 16:0, 18:0, 20:5n-3, compared to other groups, from specimens sacrificed after 1 day of exposure at all temperatures (negative scores), characterized by higher percentages of 16:0, 18:0 and 20:5n-3, and lower percentages of 14:0, 16:1n-7, 18:1n-7 and 18:1n-9, respect to other groups. PC2 separates groups on the basis of temperature of exposition: specimens exposed at 2 °C (positive scores) are characterized by higer levels of 16:1n-7, 18:1n-7 and 18:1n-9, and lower levels of 20:1n-9, 22:1n-9 and 22:6n-3, than groups exposed at 0 and 1 °C (negative scores). PC3 was associated to the time of exposition: specimens exposed at high temperatures for 1 day (positive scores, as control groups), are characterized by higher percentages of total lipids, and lower percentages of 20:1n-9, 22:1n-9 and 16:1n-7, than corresponding groups exposed at the same temperature for 5 days (scores near to 0) and 10 days (negative scores). The highest coefficients of %TL on the PC3 indicated that this parameter is the most responsible for the separation of groups.

6.5 Discussion

The results showed that the total lipid content and FA composition of muscle tissue of *T. bernacchii* are modified by temperature and time of exposure. The statistically significant reduction in total lipid content in the muscle of *T. bernacchii* in relation to the temperature of exposition (Fig. 6.1), as reported by Brodte et al. (2008), could come from a metabolic increase in fishes exposed to high temperatures (Seebaker et al. 2005; Pörtner et al. 2007). This situation could in effect determines an increased metabolic rate and consequently a rise in energy request, satisfied by triacylglycerols stored in muscle that causes a reduction in total lipid content.

High temperatures caused not only a reduction in total lipid content, but also a significant change in FAs composition. It is known that stressful habitat conditions produce oxidative stress in organisms, increasing the production of free radicals (Verdin et al. 2006; Alves de Almeida et al. 2007; Ferreira et al. 2010; Perrat et al. 2013). This entails an increase in lipid peroxidation that it is considered a useful parameter to study the response of

organisms to oxidative stress (Ghosh et al. 2013; Sattin et al. 2015). The increase in seawater temperature, to which specimens of *Trematomus bernacchii* are subjected to, could therefore produce a stressful condition, stronger than the tank conditioning, that would result in an increase in lipid peroxidation, modifying fatty acid composition, particularly throught a decrease in MUFA and PUFA content, more susceptible to the oxidation than SFA.

Taking into account he metabolic pathways of fatty acids, *in vivo* studies demonstrated an activity of elongase and desaturase enzymes involved in the FAs biosynthesis in some species of fishes (Bell et al. 2001; Izquierdo et al. 2008); Porta et al (2013) found and characterized Δ9-desaturase in *Trematomus bernacchii*, and demonstrated that this gene is expressed. Moreover, Huth and Place (2016) demonstrated an up- and down- regulation of genes involved in lipid metabolism in specimens of *Trematomus bernacchii* exposed to multiple stressors (increased temperature and pCO_2). In the light of these evidences, it is possible to discuss the obtained results considering the metabolic pathways of fatty acids. The first principal component of PCA demonstrated a contrast between 16:0 and 18:0 and their metabolites 16:1, 18:1, 20:1 and 22:1. Specimens exposed at high temperatures responded rapidly already after 1 day to the heat shock, probably modifying the metabolic pathways of palmitic and stearic acids, with an alteration of the enzimatic activity that tends to accumulate these SFAs and to reduce their metabolites. It is known that 18:1n-9 is significant to regulate membrane cell fluidity in acclimation processes (Hazel, 1983; Schünke and Wodtke, 1983; Tiku et al. 1996), therefore the significant decrease of this FA could be associated to a reduction of membrane cell fluidity to compensate the temperature increase. PC1 is dominated also by 20:5n-3 (EPA) for all treated-groups: this FA showed values significantly higher than the control groups. EPA is an important component of biological cell membranes, and its increase could indicates the need to regulate the membrane fluidity modifying the ratio between MUFA and PUFA. This regulation of membrane cell fluidity to an environmental change, i.e. homeoviscous adaptation, reflects the ability to maintain the proper fluidity of biological membranes over a certain range (Sinensky, 1974; Cossins, 1994). The PC1 also suggested that the alteration of metabolic pathways of FAs was maintained after 5 days (1 and 2 °C), and 10 days, albeit with a different proportionality between FAs compared to 1 day of exposure. FA profile of specimens exposed at 0 °C for 5 days was only sligthly modified respect to the control group, with significant changes only for total SFA (increase), 18:1n-9 and 22:6n-3 (decrease), and 20:5n-3 (increase): at this temperature specimens tryied to restore the original physiological

conditions, but after 10 days of exposure at 0 °C there is again a more significant alteration of the FA composition.

PC2, associated to the temperature of exposition, and dominated by the contrast between 16:1n-7, 18:1 and their metabolites, showed that the alteration of metabolic pathway of palmitic and stearic acid may occur differently depending on the exposure temperature, with higher level of 16:1n-7, 18:1n-7 and 18:1n-9 and lower levels of 20:1n-9 and 22:1n-9 in specimens exposed at 2 °C than other groups. Moreover, PC2 also evidenced a contrast between two important FAs for membrane fluidity: 18:1n-9 and 22:6n-3. Probably, these means that specimens exosed at 2 °C need a different change in the insaturation degree, and then a different ratio between MUFA and PUFA, than specimens exposed at 0 or 1 °C, to mantain the proper membrane functionality. Then, in agreement with literature (Brodte et al. 2008; Farkas et al. 1994), the balance between MUFA PUFA, and not only PUFA level, is probably the most important parameter to maintain the proper membrane fluidity.

Our hypothesis regarding a change in enzyme activity is also supported by Buckley and Somero (2009) and Farkas et al. (1994) who demonstrated, through cDNA microarray analysis, a certain ability of *T. bernacchi* to alter gene expression in response to heat stress, and these changes are consistent with membrane restructuring in the face of thermal stress. Moreover, it is known that in *T. bernacchii* the cellular stress response (Kültz 2005) does not involve the increase in production of heat-shock proteins, or messenger RNA, nor changes in oxygen consumption during acclimation to seawater warming (Hofmann et al. 2000; Place and Hofmann 2005; Buckley and Somero, 2009; Strobel et al. 2013; Huth and Place, 2016). Thus, the thermal tolerance induced in *T. bernacchii* may occur throught an alteration of FA composition which would fall within the broader mechanism of homeoviscous adaptation, as discussed in this work.

6.6 Conclusions

In this study, we demonstrated that *T. bernacchii* was capable to rapidly acclimatize to a heat shock. Principal component analysis helped us to hypothesize that both temperature and exposure time affect the composition of FAs in the muscle mainly through an alteration of the methabolic patways of FAs. The obtained results make a significant contribution to

the development of the general theory of adaptations of hydrobionts to environmental factors, in particular, to the temperature. This research is very important nowadays, as that there is a continuous growth of temperatures in various places around the world, especially in the areas of Antarctica and the local organisms are getting effected by the consequences of global climate change

6.7 Acknowledgements

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6.8 Figures, Tables and Supplementary materials

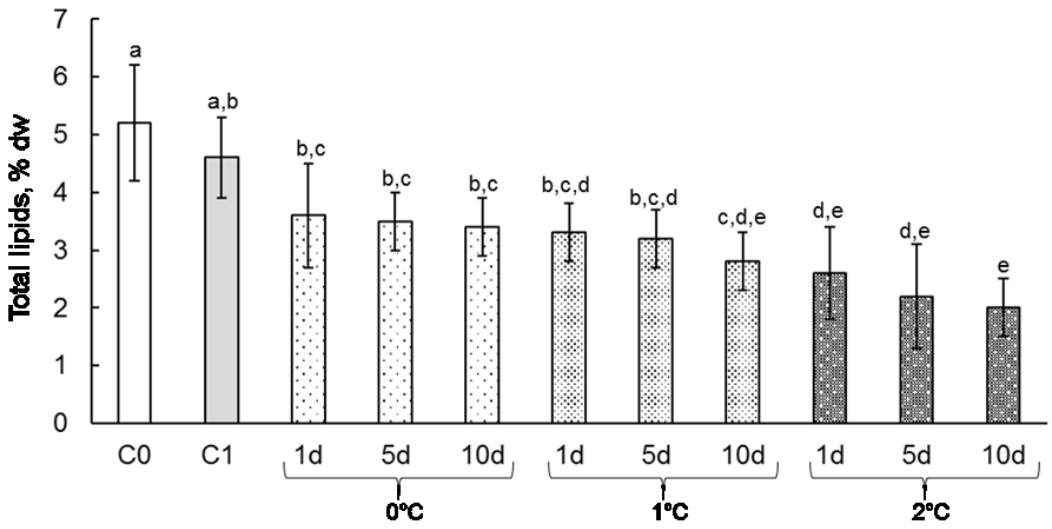

Figure 6.1. Total lipids (as % dw) of muscle of specimens of *T. bernacchii* divided *per* group. C0, control fishes as captured; C1, control fishes after acclimation; 1d, 5d and 10d indicate the time of exposure (1,5, and 10 days respectively) at the temperature indicated of 0, 1 or 2 C. Error bar represent standard deviation. One-way ANOVA test followed by multiple range test: Df 30, F = 6.59, p=0.0002.

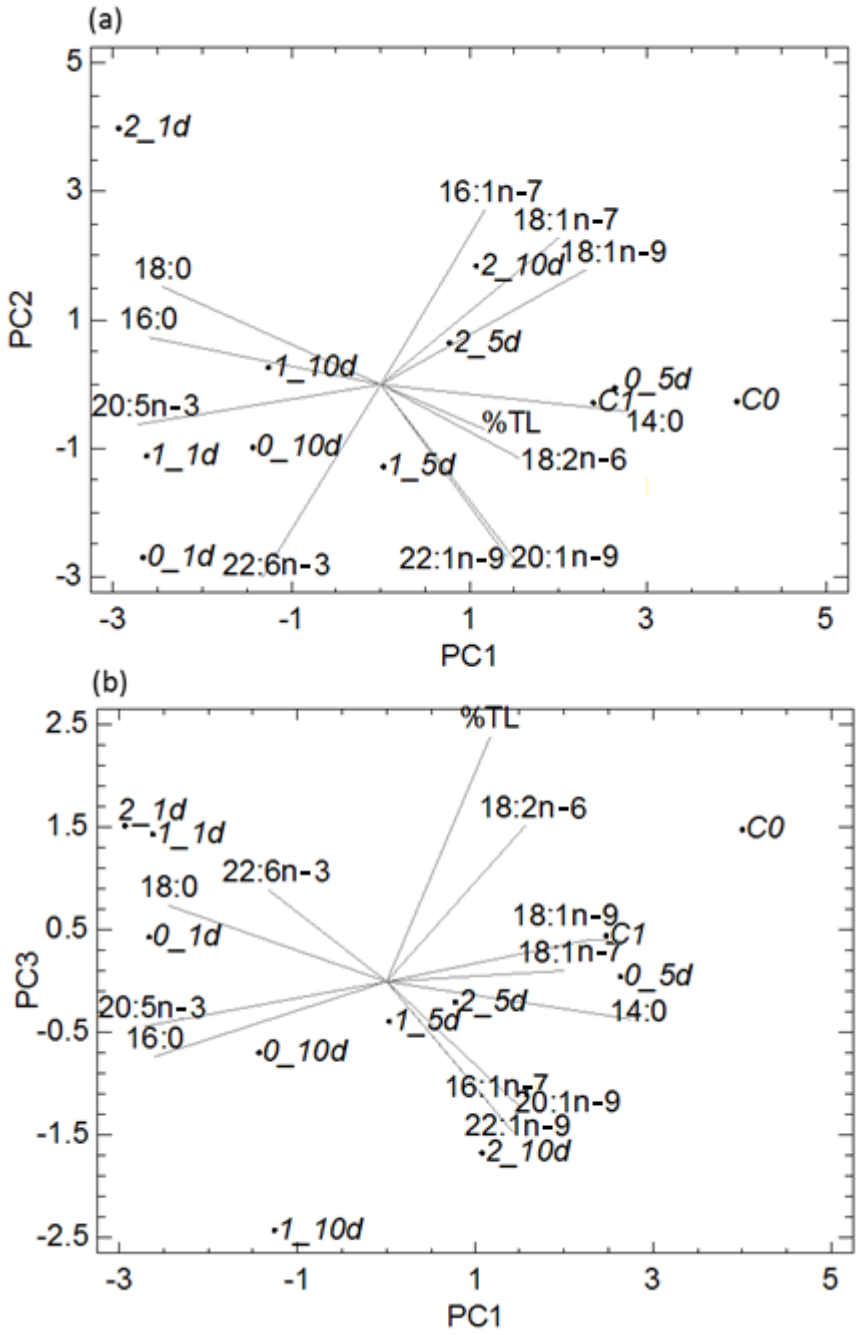

Figure 6.2. 2D biplot of PC1 *vs* PC2 (a) and PC1 *vs* PC3 (b). C0, control fishes as captured; C1, control fishes after acclimation. Other labels indicate groups exposed to the temperature indicated (0, 1 or 2 °C), followed by time of exposure; for example: 0_1d, 0_5d and 0_10d indicate animals exposed at 0 C for 1, 5, and 10 days, respectively

Table 6.1 Composition of major FAs (% *vs* total FAs, mean±SD) of muscle tissue of *T. bernacchii* held at different temperatures for different exposure times. Means within rows bearing different letters are significantly different ($p<0.05$).

[+]includes 12:0, 13:0, 15:0, 20:0, 21:0, 22:0, 23:0, 24:0

FAs	C0	C1	1day			5 days			10 days		
			0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C
14:0	8.38±0.9^{a}	7.56±0.2^{a}	5.62±0.2^{c}	5.65±0.2^{c}	5.51±0.7^{c}	8.20±0.3^{a}	7.31±0.8^{a,b}	7.10±0.5^{a,b}	6.94±0.2^{b}	6.71±0.2^{b}	6.84±0.2^{b}
16:0	9.10±0.8^{a}	9.69±0.4^{a}	11.0±0.6^{b}	11.4±0.9^{b,c}	12.1±0.9^{c}	10.2±0.4^{a,b}	10.9±0.6^{b}	10.9±0.5^{b}	11.5±0.5^{b,c}	12.1±0.5^{c}	10.2±0.4^{a,b}
18:0	1.36±0.3^{a}	1.55±0.3^{a}	2.37±0.5^{b}	3.23±0.5^{c}	4.43±0.4^{d}	1.70±0.3^{a}	2.37±0.5^{b}	2.48±0.3^{b}	2.73±0.3^{b,c}	2.63±0.4^{b,c}	1.64±0.3^{a}
Total SFAs[+]	19.5±0.6^{a}	19.6±0.7^{a}	20.1±1.1^{a,b}	21.1±1.3^{b}	22.8±0.7^{c}	20.9±0.7^{b}	21.4±0.5^{b}	21.2±1.0^{b}	22.0±0.7^{b,c}	22.3±0.9^{b,c}	19.5±0.6^{a}
16:1n-7	11.5±1.2^{a,b}	10.9±0.4^{b}	10.1±0.6^{b,c}	9.90±0.3^{c}	11.4±0.8^{a,b}	10.9±0.4^{b}	10.3±0.5^{b,c}	10.9±0.5^{b}	10.4±0.4^{b,c}	11.7±0.6^{a,b}	12.1±0.3^{a}
18:1n-9	26.7±0.9^{a}	27.4±0.5^{a}	18.7±0.5^{e}	20.3±0.6^{d}	23.9±0.9^{c}	25.4±0.8^{b}	23.1±0.7^{c}	27.0±0.5^{a}	20.0±0.5^{d}	19.8±0.8^{d}	27.1±0.5^{a}
18:1n-7	6.52±0.3^{a}	6.0±0.3^{a}	4.52±0.2^{c}	5.16±0.2^{b}	6.15±0.2^{a}	6.47±0.1^{a}	5.48±0.1^{b}	5.56±0.2^{b}	5.58±0.3^{b}	5.80±0.1^{b}	6.01±0.2^{a}
20:1n-9	5.59±0.5^{a}	5.70±0.3^{a}	5.46±0.5^{a}	4.65±0.5^{b}	3.33±0.2^{c}	5.50±0.3^{a}	5.76±0.6^{a}	5.20±0.3^{a,b}	5.34±0.5^{a,b}	5.67±0.4^{a}	4.93±0.2^{b}
22:1n-9	2.19±0.4^{a,b}	2.17±0.2^{a}	2.13±0.2^{a}	1.94±0.1^{a,b}	1.15±0.1^{c}	2.29±0.2^{a}	2.20±0.4^{a,b}	2.06±0.3^{a,b}	2.20±0.2^{a}	2.29±0.3^{a}	2.06±0.3^{a,b}
24:1n-9	0.99±0.2	0.98±0.2	0.91±0.1	0.81±0.1	0.70±0.1	1.08±0.1	0.89±0.1	0.89±0.1	0.94±0.1	0.85±0.1	0.85±0.1
Total MUFAs[*]	54.4±0.7^{a}	53.8±0.5^{a}	42.8±0.4^{e}	43.8±1.5^{d,e}	47.5±1.7^{c}	52.8±1.1^{a,b}	48.8±1.2^{c}	52.5±0.4^{b}	45.4±0.6^{d}	47.1±0.6^{c}	54.0±0.5^{a}
18:3n-3	0.83±0.2	0.67±0.1	0.99±0.2	0.93±0.1	0.82±0.1	0.80±0.1	0.75±0.1	0.74±0.1	0.86±0.1	0.70±0.1	0.70±0.1
20:5n-3	10.3±0.4^{a}	11.0±0.4^{a}	16.8±1.0^{f}	16.0±1.1^{e}	14.7±0.6^{d}	12.0±0.4^{b}	12.4±0.6^{b}	12.4±0.9^{b}	15.2±0.4^{d,e}	15.4±0.4^{d,e}	13.2±0.4^{c}
22:6n-3	10.9±1.3^{c}	10.7±0.6^{c}	14.5±0.4^{a}	13.1±1.1^{b}	9.43±0.9^{d,e}	9.16±0.5^{e}	12.3±1.0^{b}	8.71±0.5^{e}	12.1±0.8^{b}	10.1±0.4^{c,d}	8.57±0.6^{e}
Total n-3 PUFAs°	22.2±1.2^{a}	22.4±0.5^{a}	32.5±1.3^{f}	30.3±1.2^{d}	25.1±1.1^{b}	22.1±0.9^{a}	25.5±1.1^{b}	21.9±0.8^{a}	28.4±1.0^{c}	26.3±0.7^{b}	22.6±0.8^{a}
18:2n-6	2.45±0.4^{a,b}	2.15±0.2^{a}	2.19±0.3^{a}	2.32±0.2^{a,b}	2.07±0.1^{a}	2.45±0.1^{b}	2.20±0.1^{a}	2.36±0.1^{a,b}	2.15±0.1^{a}	2.03±0.1^{a}	2.08±0.1^{a}
20:4n-6	0.58±0.2^{a}	0.52±0.1^{a}	1.15±0.1^{c}	1.33±0.2^{c}	1.19±0.2^{c}	0.85±0.1^{b}	1.08±0.1^{c}	0.96±0.1^{b,c}	0.86±0.1^{b}	0.99±0.1^{b,c}	0.81±0.1^{b}
Total n-6 PUFAs^	3.80±0.8^{a}	4.04±0.3^{a}	4.36±0.1^{b}	4.69±0.2^{c}	4.27±0.3^{b}	4.08±0.1^{a}	4.13±0.2^{a}	4.13±0.1^{a}	4.00±0.1^{a}	3.97±0.1^{a}	3.77±0.2^{a}
Total PUFAs°^	26.0±0.7^{a}	26.5±0.5^{a}	36.9±1.4^{d}	35.0±1.2^{d}	29.5±1.2^{b}	26.2±1.0^{a}	29.7±1.2^{b}	26.1±0.8^{a}	32.4±1.0^{c}	30.3±0.6^{b}	26.4±0.8^{a}

[*]includes 14:1n-5, 15:1n-5, 17:1n-7
°includes 20:3n-3
^includes 18:3n-6, 20:2n-6, 20:3n-6

Table 6.2. Principal component analysis. Eigenvalues, explained and cumulative variance, loadings of the variables for the first three PCs.

	Principal Components		
	1	2	3
Variance explained			
Eigenvalues	5.642	3.106	1.637
% of variance	47.02	25.88	13.64
Cumulative %	47.02	72.90	86.54
Factor loadings			
14:0	0.395	-0.063	-0.094
16:0	-0.373	0.110	-0.187
18:0	-0.352	0.226	0.186
16:1n-7	0.168	0.403	-0.305
18:1n-9	0.332	0.266	0.102
18:1n-7	0.289	0.339	0.024
20:1n-9	0.219	-0.412	-0.315
22:1n-9	0.205	-0.405	-0.375
18:2n-6	0.226	-0.173	0.384
20:5n-3	-0.392	-0.094	-0.112
22:6n-3	-0.190	-0.448	0.226
%TL	0.168	-0.106	0.605

Table S6.1. FAs composition (% *vs* total FAs, mean±SD) of the muscle tissue of *T. bernacchii* held at different temperatures for different time.

FAs	C0	C1	1day			5 days			10 days		
			0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C
12:0	0.12±0.1	0.08±0.1	0.23±0.1	0.12±0.1	0.11±0.1	0.17±0.1	0.17±0.1	0.18±0.1	0.16±0.1	0.18±0.1	0.13±0.1
13:0	0.03±0.1	0.02±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1	0.03±0.1
14:0	8.38±0.9^{a}	7.56±0.2^{a}	5.62±0.2^{c}	5.65±0.2^{c}	5.51±0.7^{c}	8.20±0.3^{a}	7.31±0.8^{a,b}	7.10±0.5^{a,b}	6.94±0.2^{b}	6.71±0.2^{b}	6.84±0.2^{b}
15:0	0.29±0.1	0.37±0.1	0.47±0.1	0.40±0.1	0.34±0.1	0.39±0.1	0.38±0.1	0.35±0.1	0.39±0.1	0.45±0.1	0.37±0.1
16:0	9.10±0.8^{a}	9.69±0.4^{a}	11.0±0.6^{b}	11.4±0.9^{b,c}	12.1±0.9^{c}	10.2±0.4^{a,b}	10.9±0.6^{b}	10.9±0.5^{b}	11.5±0.5^{b,c}	12.1±0.5^{c}	10.2±0.4^{a,b}
17:0	0.10±0.1	0.10±0.1	0.23±0.1	0.22±0.1	0.21±0.1	0.15±0.1	0.17±0.1	0.15±0.1	0.21±0.1	0.25±0.1	0.13±0.1
18:0	1.36±0.3^{a}	1.55±0.3^{a}	2.37±0.5^{b}	3.23±0.5^{c}	4.43±0.4^{d}	1.70±0.3^{a}	2.37±0.5^{b}	2.48±0.3^{b}	2.73±0.3^{b,c}	2.63±0.4^{b,c}	1.64±0.3^{a}
20:0	0.05±0.01	0.19±0.01	0.18±0.01	0.14±0.02	0.09±0.02	0.08±0.01	0.11±0.02	0.08±0.01	0.10±0.01	0.10±0.01	0.10±0.01
21:0	0.03±0.01	0.02±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01
22:0	0.09±0.01	0.07±0.02	0.06±0.01	0.08±0.01	0.08±0.01	0.06±0.01	0.04±0.01	0.06±0.01	0.06±0.01	0.05±0.01	0.08±0.01
23:0	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.04±0.01	0.02±0.01	0.02±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0.03±0.01
24:0	0.07±0.01	0.04±0.01	0.06±0.03	0.04±0.01	0.08±0.01	0.03±0.01	0.03±0.01	0.02±0.01	0.04±0.01	0.03±0.01	0.02±0.01
Total SFAs	19.5±0.6^{a}	19.6±0.7^{a}	20.1±1.1^{a,b}	21.1±1.3^{b}	22.8±0.7^{c}	20.9±0.7^{b}	21.4±0.5^{b}	21.2±1.0^{b}	22.0±0.7^{b,c}	22.3±0.9^{b,c}	19.5±0.6^{a}
14:1n-5	0.40±0.1	0.58±0.1	0.37±0.1	0.34±0.1	0.34±0.1	0.56±0.1	0.40±0.1	0.32±0.1	0.40±0.1	0.45±0.1	0.33±0.1
15:1n-5	0.01±0.001	0.01±0.01	0.02±0.002	0.02±0.008	0.04±0.01	0.02±0.002	0.02±0.004	0.02±0.007	0.02±0.003	0.02±0.001	0.02±0.006
16:1n-7	11.5±1.2^{a,b}	10.9±0.4^{b}	10.1±0.6^{b,c}	9.90±0.3^{c}	11.4±0.8^{a,b}	10.9±0.4^{b}	10.3±0.5^{b,c}	10.9±0.5^{b}	10.4±0.4^{b,c}	11.7±0.6^{a,b}	12.1±0.3^{a}
17:1n-7	0.43±0.1	0.49±0.1	0.61±0.1	0.62±0.1	0.51±0.1	0.56±0.1	0.54±0.1	0.57±0.1	0.55±0.1	0.64±0.1	0.58±0.1
18:1n-9	26.7±0.9^{a}	27.4±0.5^{a}	18.7±0.5^{e}	20.3±0.6^{d}	23.9±0.9^{c}	25.4±0.8^{b}	23.1±0.7^{c}	27.0±0.5^{a}	20.0±0.5^{d}	19.8±0.8^{d}	27.1±0.5^{a}
18:1n-7	6.52±0.3^{a}	6.0±0.3^{a}	4.52±0.2^{c}	5.16±0.2^{b}	6.15±0.2^{a}	6.47±0.1^{a}	5.48±0.1^{b}	5.56±0.2^{b}	5.58±0.3^{b}	5.80±0.1^{b}	6.01±0.2^{a}
20:1n-9	5.59±0.5^{a}	5.70±0.3^{a}	5.46±0.5^{a}	4.65±0.5^{b}	3.33±0.2^{c}	5.50±0.3^{a}	5.76±0.6^{a}	5.20±0.3^{a,b}	5.34±0.5^{a,b}	5.67±0.4^{a}	4.93±0.2^{b}
22:1n-9	2.19±0.4^{a,b}	2.17±0.2^{a}	2.13±0.2^{a}	1.94±0.1^{a,b}	1.15±0.1^{c}	2.29±0.2^{a}	2.20±0.4^{a,b}	2.06±0.3^{a,b}	2.20±0.2^{a}	2.29±0.3^{a}	2.06±0.3^{a,b}
24:1n-9	0.99±0.2	0.98±0.2	0.91±0.1	0.81±0.1	0.70±0.1	1.08±0.1	0.89±0.1	0.89±0.1	0.94±0.1	0.85±0.1	0.85±0.1
Total MUFAs	54.4±0.7^{a}	53.8±0.5^{a}	42.8±0.4^{e}	43.8±1.5^{d,e}	47.5±1.7^{c}	52.8±1.1^{a,b}	48.8±1.2^{c}	52.5±0.4^{b}	45.4±0.6^{d}	47.1±0.6^{c}	54.0±0.5^{a}
18:3n-3	0.83±0.2	0.67±0.1	0.99±0.2	0.93±0.1	0.82±0.1	0.80±0.1	0.75±0.1	0.74±0.1	0.86±0.1	0.70±0.1	0.70±0.1
20:3n-3	0.09±0.01	0.11±0.01	0.20±0.4	0.17±0.02	0.15±0.01	0.12±0.01	0.11±0.01	0.14±0.01	0.16±0.01	0.08±0.01	0.16±0.01
20:5n-3	10.3±0.4^{a}	11.0±0.4^{a}	16.8±1.0^{f}	16.0±1.1^{e}	14.7±0.6^{d}	12.0±0.4^{b}	12.4±0.6^{b}	12.4±0.9^{b}	15.2±0.4^{d,e}	15.4±0.4^{d,e}	13.2±0.4^{c}
22:6n-3	10.9±1.3^{c}	10.7±0.6^{c}	14.5±0.4^{a}	13.1±1.1^{b}	9.43±0.9^{d,e}	9.16±0.5^{e}	12.3±1.0^{b}	8.71±0.5^{e}	12.1±0.8^{b}	10.1±0.4^{c,d}	8.57±0.6^{e}
Total n-3 PUFAs	22.2±1.2^{a}	22.4±0.5^{a}	32.5±1.3^{f}	30.3±1.2^{d}	25.1±1.1^{b}	22.1±0.9^{a}	25.5±1.1^{b}	21.9±0.8^{a}	28.4±1.0^{c}	26.3±0.7^{b}	22.6±0.8^{a}
18:2n-6	2.45±0.4^{a,b}	2.15±0.2^{a}	2.19±0.3^{a}	2.32±0.2^{a,b}	2.07±0.1^{a}	2.45±0.1^{b}	2.20±0.1^{a}	2.36±0.1^{a,b}	2.15±0.1^{a}	2.03±0.1^{a}	2.08±0.1^{a}
18:3n-6	0.19±0.1	0.18±0.1	0.26±0.1	0.24±0.2	0.38±0.3	0.20±0.1	0.24±0.4	0.31±0.3	0.29±0.2	0.28±0.4	0.27±0.2
20:2n-6	0.31±0.1	0.65±0.1	0.57±0.2	0.57±0.1	0.46±0.1	0.42±0.1	0.42±0.1	0.32±1	0.51±0.1	0.50±0.1	0.45±0.1
20:3n-6	0.27±0.2	0.14±0.1	0.17±0.1	0.20±0.1	0.16±0.1	0.16±0.1	0.14±0.1	0.18±0.1	0.19±0.1	0.16±0.1	0.15±0.1
20:4n-6	0.58±0.2^{a}	0.52±0.1^{a}	1.15±0.1^{c}	1.33±0.2^{c}	1.19±0.2^{c}	0.85±0.1^{b}	1.08±0.1^{c}	0.96±0.1^{b,c}	0.86±0.1^{b}	0.99±0.1^{b,c}	0.81±0.1^{b}
Total n-6 PUFAs	3.80±0.8^{a}	4.04±0.3^{a}	4.36±0.1^{b}	4.69±0.2^{c}	4.27±0.3^{b}	4.08±0.1^{a}	4.13±0.2^{a}	4.13±0.1^{a}	4.00±0.1^{a}	3.97±0.1^{a}	3.77±0.2^{a}
16:2n-7	0.08±0.03	0.05±0.01	0.06±0.01	0.08±0.01	0.09±0.02	0.07±0.01	0.06±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.05±0.01
Total PUFAs	26.0±0.7^{a}	26.5±0.5^{a}	36.9±1.4^{d}	35.0±1.2^{d}	29.5±1.2^{b}	26.2±1.0^{a}	29.7±1.2^{b}	26.1±0.8^{a}	32.4±1.0^{c}	30.3±0.6^{b}	26.4±0.8^{a}

Means within rows bearing different letters are significantly different ($p<0.05$).

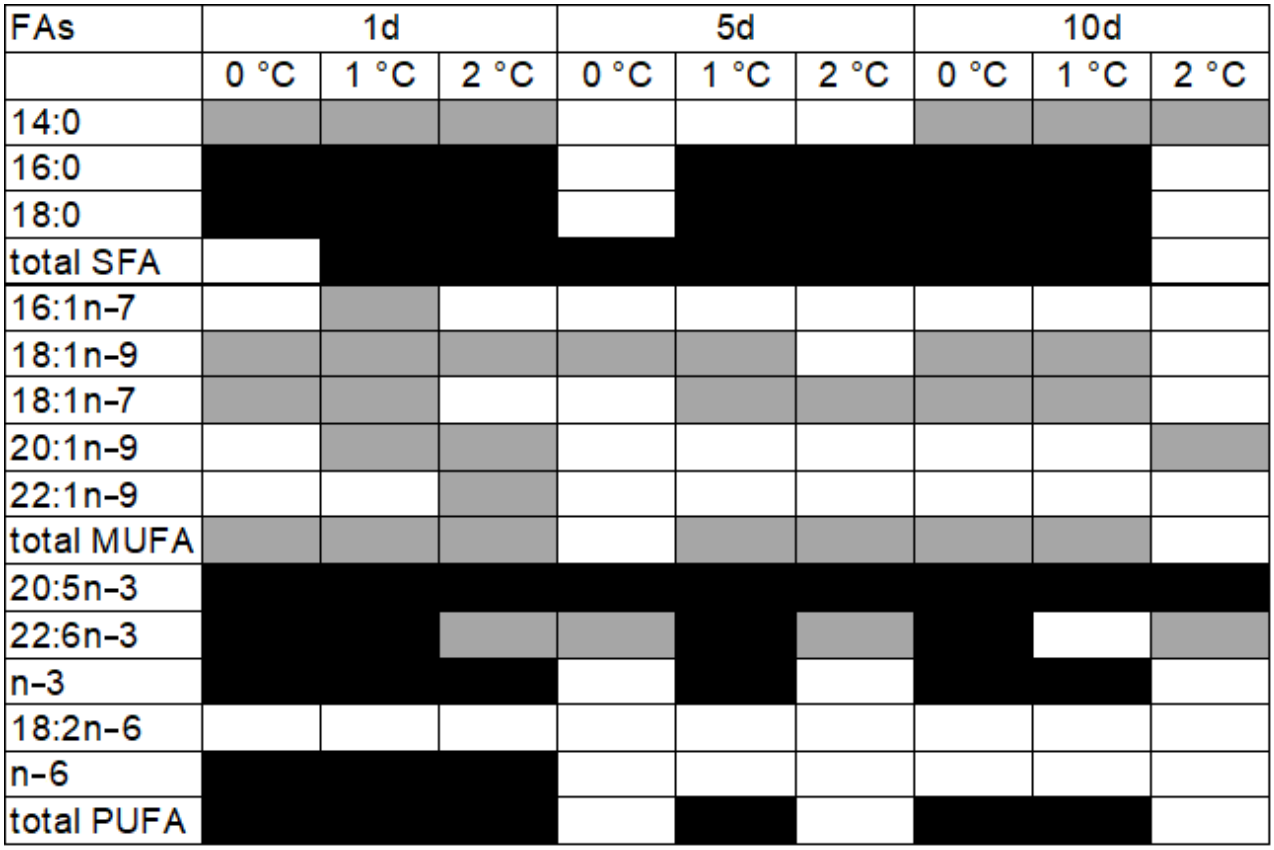

Fig. S6.1 Influence of temperature and time of exposure on the percentage of FAs of muscle of *T. bernacchii*. Only statistically significant changes ($p<0.05$) respect to control groups (grey, decrease; black, increase; white no significative change) were reported.

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7 Gas Chromatography-Mass Spectrometry analysis on effects of thermal shock on the fatty acid composition of the gills of the Antarctic teleost *Trematomus bernacchii*

7.1 Abstract

Due to rising temperatures in the Antarctic seawater, there is an increasing interest to investigate the capacities for inducing a temperature acclimation response in Antarctic organisms. In this study, we analyzed the effect of a thermal shock on the fatty acid (FA) composition of gills of the Antarctic notothenioid *Trematomus bernacchii*. To perform the quantification of fatty acids in gills, we applied an analytical method based on a fast microwave-assisted extraction (MAE) of lipids from a lyophilized sample, a base-catalyzed trans-esterification of lipid extract to obtain fatty acids methyl esters (FAMEs), and their separation and identification by Gas Chromatography-Mass Spectrometry. The percentage of lipids extracted with MAE preceded by sample lyophilization, 0.6±0.3% ww (wet weight), was consistent with lipids obtained with the official Bligh & Dyer method, 0.8±0.3% ww. These data are among the first ever published for *T. bernacchii*. Specimens, caught in Terra Nova Bay (Ross Sea), were held in different tanks at 0, +1 and +2 °C, for 1, 5 and 10 days. In general, thermal shock produced an alteration in total lipid content, an increase in the percentage of saturated FAs, a decrease in mono-unsaturated FAs, and a variable response of poly-unsaturated FAs (an increase in specimens exposed to 0 and +1 °C, a decrease in those exposed to +2 °C). A chemometric approach based on Principal Component Analysis indicated that temperature and exposure time influenced the composition of FAs in the gills, probably through an alteration of the metabolic pathways of FAs. This is to our knowledge the first study ever published on FA composition of total lipids in gills of *T. bernacchii* exposed to high temperatures. This study demonstrated that *T. bernacchii* is capable of rapidly acclimating to a thermal shock, and contributes to increasing knowledge on the effect of temperature on Antarctic notothenioids.

7.2 Introduction

Global climate change is becoming a very important problem for life, leading to significant alteration in the physiological and biogeochemical processes of organisms (Hixson and Arts 2016; Pörtner et al. 2005; Teoh et al. 2010; Tomanek 2008). The Intergovernmental Panel on Climate Change IPCC (2014) predicted alterations in the global mean surface temperature of 3-6 °C by the year 2100. Also polar environments are affected by rising temperatures (Constable et al. 2014; Smetacek and Nicol 2005): in the Ross Sea region, Antarctica (average temperature of -1.87 °C), shelf water warming up to +0.8-+1.4 °C is predicted by 2200 (Timmermann and Hellmer 2013). Considering the possible impact of climate changes on the Antarctic biota, it is crucial to characterize the ability of Antarctic fishes to deal with changes in water temperature, since the migration to more favorable environments is highly unlikely for these species (Huth and Place 2016).

Polar fishes are characterized by a high density of mitochondria and lipid droplets that cover important physiological roles (Grim et al. 2010; Machado et al. 2014; Sidell 1998). These two particular characteristics indicate that fishes of this particular region use lipid catabolism as the principal source of energy (Colella et al. 2000; Sidell and Hazel 2002). A further adaptation of polar fishes at the cold environment is the high content of unsaturated fatty acids in the biological membrane. This phenomenon, called “homeoviscous adaptation”, is essential for maintaining the correct fluidity of membrane necessary for its biological functions (Roche and Pérès 1984; Sinensky 1974).

Fishes of the suborder Notothenioidei comprise the largest fraction of fishes endemic of Antarctic waters (Eastman et al. 1993); they live permanently at low temperatures (about -1.8 °C) and some species are considered highly stenothermal, being highly specialized to survive at low temperatures (Clarke 1991; Eastman et al. 1990; Pörtner and Playle 2007). Several studies aimed to explore warm acclimation response in Antarctic notothenioid fishes such as *Notothenia coriiceps* and/or *Notothenia rossii* (Forgati et al. 2017; Klein et al. 2017; Machado et al. 2014; Mueller et al. 2014; Souza et al. 2018; Strobel et al. 2013), *Pagothenia borchgrevinki* (Carney Almroth et al. 2015; Franklin et al. 2007, 2001; Lowe et al. 2005; Podrabsky and Somero 2006; Ryan 1995; Seebacher et al. 2005) or other species of fishes such as the icefish *Chionodraco rastrospinosus* (Mueller et al. 2014) or the eelpout *Pachycara brachycephalum* (Windisch et al. 2014). These studies demonstrated that some species are more tolerant to warmer temperatures due to climate change and that fishes did

not lose the capacity to respond to environmental changes, although they present a narrow window of thermal tolerance.

The notothenioid teleost *Trematomus bernacchii* (Boulenger, 1902) is one of the most abundant fishes in Antarctic seawater (Near and Cheng 2008), and for this reason it is often used as a reliable bio-indicator for environmental studies (Barbaro et al. 2016; Bargagli et al. 1998; Borghesi et al. 2008; Corsolini et al. 2006; Ghosh et al. 2013; Illuminati et al. 2010; Regoli et al. 2005). Starting from Somero and DeVries (1967), several studies focused on thermal acclimation response of *T. bernacchii*, considering numerous parameters, such as antioxidant defence system (Enzor and Place 2014), transcriptome wide analysis and gene expression (Buckley et al. 2004; Huth and Place 2016, 2013; Place and Hofmann 2005; Tolomeo et al. 2016), oxygen consumption and cardiorespiratory performance (Jayasundara et al. 2013; Weinstein and Somero 1998), temperature survival and critical thermal maximum (Bilyk and DeVries 2011; Podrabsky and Somero 2006), physiological performances (Davis et al. 2018; Sandersfeld et al. 2015), cortisol levels (Hudson et al. 2008), cellular apoptosis (Sleadd 2014). Moreover, fatty acid composition of tissues was explored in *T. bernacchii* exposed to thermal shock; Malekar et al. (2018) demonstrated that a thermal acclimation at 6 °C resulted in an increase of saturated fatty acids and a decline in unsaturated fatty acids in membrane of liver tissue, showing a homeoviscous response to maintain the proper membrane functionality. Truzzi et al. (2018), demonstrated a capability of *T. bernacchii* to acclimatize to a heat shock modifying the fatty acid composition of the muscle of the fish, probably through a modification of metabolic pathways of FA. Few studies on warm acclimation of *T. bernacchii* have considered gill tissue, the fundamental organ for respiration in fish, where the exchange of the respiratory gases (O_2 and CO_2) takes place (Perry and Gilmour 2002). In this tissue, especially in Antarctic fishes, lipids play an important role in increasing the dissolution ratio and the uptake-efficiency of oxygen (Sidell 1998). It is known that an icrease of environmental temperature leads to a reduction of oxygen availability in marine organisms (decreasing of PO_2 in blood) that determines a deficiency in oxygen bioavailability for tissues (Pörtner and Knust 2007). Studies on warm acclimation of *T. bernacchii* exploring gill tissue focused on transcriptome wide analysis or gene expression (Buckley and Somero 2009; Enzor et al. 2017; Huth and Place 2016, 2013), antioxidant defence system (Enzor and Place 2014), Na^+/K^+-ATPase activity and osmoregulation (Brauer et al. 2005; Enzor et al. 2017; Gonzalez-Cabrera et al. 1995; Guynn et al. 2002; Morrison et al. 2006). These studies demonstrated, in general, a rapid cellular stress response, an ability to alter gene expression, an increase of the Na^+/K^+-ATPase

activity and an hypo-osmoregulation, in response to heat stress. To our knowledge, there are no studies regarding the fatty acid composition of total lipids of gills of *T. bernacchii* and the relation of the fatty acid profile with a thermal shock.

Due to the important roles of lipids in polar fishes, being the primary substrate of aerobic metabolism and essential structural components of cells, the aim of this study is to investigate the warm acclimation response of *T. bernacchii* exposed to a thermal shock by studying the total fatty acids composition of gills tissue of the fish. The expected outcome (also in light of previous studies found in the literature on *T. bernacchii* exposed to a heat shock) was a modification of FA profile, due to a rapid acclimation process. To our knowledge, this is the first attempt to relate changes in total fatty acid composition in gills to an acclimation response following a thermal stress in *T. bernacchii*, whereas similar studies were performed in other Antarctic or temperate species (Ackman 1989; Brodte et al. 2008).

To achieve this goal, we applied an analytical methodology already optimized for muscle tissue of fish (Truzzi et al. 2017) based on microwave-assisted extraction (MAE) of lipids from a lyophilized sample, derivatization of fatty acids via transesterification with $NaOCH_3$ to obtain FAMEs, and FAMEs separation and identification by Gas chromatography-Mass spectrometry. MAE is a well-suited alternative to conventional methods for the extraction of lipids from different matrices (Mahesar et al. 2008; Ramalhosa et al. 2012; Virot et al. 2007) as it allows a fast extraction that exposes the sample to relatively high temperatures for a very short time, and it is a solvent-sparing technique. The method has been adapted in order to reduce the extraction time as well as to enhance the efficiency of the extraction, in comparison with the official Bligh & Dyer method (1959).

After more than 50 years of research focused on the adaptation of notothenioids to climate warming, there are still many questions that remain to be answered (Beers and Jayasundara 2015). This study provides important novel information on the acclimation of Antarctic fishes to a thermal shock, and it may help to better understand the response of the Antarctic fish to rising temperatures.

7.3 Material and methods

7.3.1 Chemicals

All solvents and reagents were of HPLC grade: acetone, petroleum ether 35-60 °C, chloroform, and anydrous sodium sulphate (Carlo Erba, Milano, Italy); *n*-heptane and methanol (Baker, Philipsburg, NJ, USA); sodium methoxide for synthesis (≥ 97%, Merck, Hohenbrunn, Germany); sodium hydrogen sulfate anhydrous, extra pure (Scharlau, Sentmenat, Spain); 37-component FAME mix (≥ 99%, Supelco, Bellefonte, PA, USA); methyl ester of nonadecanoic acid used as internal standard (99.6%, Dr. Ehrenstorfer GmbH, Germany).

7.3.2 Sample collection and experimental design

During the 28th Italian Antarctic Expedition (austral summer 2014–2015), 66 sexually mature specimens of *T. bernacchii* (weight 136-333 g, length 22-30 cm) were caught by a fishing rod in Tethys Bay (74°42'052" S, 164°02'267" E), Ross Sea, Northern Victoria Land, at the depth of ~30 m. The seawater temperature at the moment of the capture (October 2014) was -1.7 °C, consistent with temperatures measured during the austral summer of other years (Illuminati et al, 2017), close to the ‘‘Mario Zucchelli’’ Station. The experimental design was applied as in Truzzi et al. (2018). Briefly, six specimens were sacrificed just after the capture and used as “control group” (C0). The other fishes were tank-acclimated under ambient conditions (-1.8 °C, controlled to within 0.1 °C) for 10 days in a flow-through seawater fish tank (1000 L); after that, they were directly kept in closed circuit tanks (200 L) at 0, +1 or +2 °C (controlled to within 0.1 °C), where air was continuously infused, and sacrificed after 1, 5 and 10 days. Six fishes were still maintained at -1.8 °C for other 10 days, and then sacrificed, and used as “control group after acclimation” (C1). For each treated group, 6 specimens were used. During both the acclimation and the experiment period, fishes were fed with chopped fish muscle ad libitum (no substantial differences between groups in the amount of fish eaten were evidenced). Sacrificed fishes were rapidly measured and weighed. Overall mean length was 26±2 cm (range 22-30 cm); overall mean body weight was 250±66 g (range 113-367 g) (see Table S1 for complete dataset). No statistically significant differences were obtained for body length and weight ($P>0.05$). Tissues were

quickly excised, placed into cryotubes and snap-frozen in liquid nitrogen, and later stored at −80°C until fatty acid analysis.

7.3.3. Ethics

All procedures involving animals were conducted in line with Italian legislation on experimental animals and were carried out on behalf of the Italian Ministry of Foreign Affairs. The Code of Conduct of the Scientific Committee on Antarctic Research (SCAR) was followed for the handling and sacrificing of animals.

7.3.4 Lipid extraction and fatty acid analysis

For gills analysis, only gill filaments were considered, after excluding the gill arches; left and right gill filaments were minced, homogenized (homogenizer MZ 4110, DCG Eltronic), and divided into aliquots of about 1 g. Analyses were carried out on three aliquots *per* fish. Lipid extraction was carried out on lyophilized samples (Edwards EF4, Crawley, Sussex, England) by Microwave-Assisted Extraction (MAE) according to Truzzi et al (2017). Briefly, tissues were accurately weighed and freeze-dried (Edwards EF4 modulyo, Crawley, Sussex, England) until constant weight (±0.2 mg). Samples were transferred in a Teflon extraction vessel of a Microwave Accelerated Reaction System, MARS-X, 1500 W (CEM, Mathews, NC, USA) with 20 mL of petroleum ether (35-60 °C): acetone (2:1, v/v, Carlo Erba, Milano, Italy), to perform a Microwave-Assisted Extraction. The operational parameters were: magnetron power 100%; time to reach settings 10 min; extraction time 20 min; extraction temperature 90 °C and maximum vessel pressure cut off $1.38 \cdot 10^6$ Pa. After cooling, the extract was filtered through Whatman GF/C filter paper (England) filled with Na_2SO_4. The extracts were evaporated under laminar flow inert gas (N_2) until constant weight. After drying, the mass of extracted lipids was determined.

FAMEs were prepared according to Canonico et al. (2016), using the methyl ester of nonadecanoic acid (19:0) as the internal standard. FAMEs were determined on an Agilent-6890 GC System coupled with an Agilent-5973N quadrupole Mass Selective Detector (MSD). A CPS ANALITICA CC-wax-MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column was used to separate FAMEs. Instrumental conditions were as reported by

Truzzi et al. (2017): sample injections of 1 µL were made in a split mode ratio (1:5 or 1:10) using a glass cup liner (Agilent Liner, slittess, double taper 5583-4705). The inlet temperature was kept at 250 °C. Helium carrier gas (99.9999%, SOL, Italy) (8.0 psi) was used at a flow rate of 1 mL min^{-1}. The oven temperature started at 100 °C for 1 min, and it was subsequently increased to 150 °C at the rate of 25 °C min^{-1}, to 200 °C at the rate of 5 °C min^{-1} and to 230 °C at the rate of 1 °C min^{-1}, for a total run time of 43 min. Data collection, identification, and quantification of FAs were as reported in Truzzi et al. (2017). Retention time and mass spectra of 37-component FAME Mix standard were used to confirm the NIST identification of FAs in the sample. For each sample, at least three runs were performed on the GC-MS. Method performance was similar to that obtained in the analysis of FAMEs obtained from muscle tissue of *T. bernacchii*: the linearity was checked up to about 320 µg mL^{-1}, the limit of detection (LOD), and limit of quantification (LOQ), calculated as reported in Truzzi et al. (2014), ranged from ~4 µg mL^{-1} to ~22 µg mL^{-1}, and from ~13 µg mL^{-1} to ~66 µg mL^{-1}, respectively (Truzzi et al. 2017). Moreover, the method showed a good accuracy and precision.

7.3.5 Statistical analysis

To compare body length and weight of specimens, total lipid percentage and FAs composition between groups, the one-way-ANOVA test, followed by the multiple range test (Daniel and Cross 2013) were performed after testing the homogeneity of variance with Levene's test. Significant differences were evaluated at the 95% confidence level. Principal Component Analysis (PCA) was carried out on standardized data; significant components were obtained through the Wold cross-validation procedure (Wold 1978) ANOVA test and PCA were performed using Statgraphics Plus 5.1 (2009, Manugistics Inc., Rockville, Maryland, USA).

7.4 Results and Discussion

7.4.1 Optimization of lipid extraction

The percentage of water in the gills of *T. bernacchii* was high (81±1%) and justified the introduction of a lyophilization step that allows a complete loss of water. It is known, indeed, that the presence of traces of water leads to poor recoveries of FAMEs, and water can cause some problems during the extraction of lipids, producing co-extractables which complicate the analysis (Basconcillo and McCarry 2008; Juárez et al. 2008). The percentage of lipids extracted with MAE preceded by sample lyophilization was 0.6±0.3% ww (wet weight), significantly higher ($p < 0.05$) than the percentage obtained without the lyophilization step, 0.3±0.2% ww (Table 7.1). To test the accuracy of MAE method, a lipid extraction from gill sample was performed with the official Bligh & Dyer extraction method (1959). The percentage of lipids obtained with the official method was 0.8±0.3% ww (Table 7.1), not significantly different ($p > 0.05$) from the lipid extraction obtained with MAE on freeze-dried samples. These data are among the first ever published for *T. bernacchii*, and it is therefore not possible to make any comparison. In any case, our results are in agreement with literature data regarding lipid in gills of notothenioid fish other than *T. bernacchii* (Table 7.2).

Figure 7.1 shows an example of chromatograms of FAMEs obtained from lipid fractions of gills extracted with MAE (Fig. 7.1a) or Blig & Dyer (Fig. 7.1b) methods. No overlapping between peaks was noted with the MAE extraction, whereas broad peaks, overlapping, and substances different from FAMEs (in addition to FAMEs) were observed with the Bligh & Dyer method.

7.4.2 Lipid content

The percentage of dry weight (dw) in the gill of *T. bernacchii* varied from 17% to 20% (mean 19%±1%), and no statistically significant differences were recorded between specimens of different groups. Fig. 7.2 shows total lipid percentages (%TL) *vs* dw of the gill tissue of specimens of *T. bernacchii* exposed to different temperatures. Differences in total lipids percentage of the two control groups (fishes as captured, C0, and after acclimation, C1) were not statistically significant, as well as differences between control groups and specimens exposed to 0, 1 and 2 °C for 1 and 5 days, apart from the specimens exposed to

1 °C for 5 days, that showed a %TL statistically lower than control groups. Specimens exposed to 0 °C for 10 days showed an increasing trend with respect to control groups, whereas those exposed to 1 and 2 °C for 10 days showed a %TL statistically higher than other groups. These results showed that the gills were able to maintain the same %TL up to 5 days of exposure to high temperatures (Fig. 7.2): probably the fish was initially able to compensate for the increase in oxygen request due to higher temperatures (Grim et al. 2010; Jayasundara et al. 2013; Sidell 1998). Successively, with longer exposure time to high temperatures, the fish tried to compensate the greater oxygen demand with an increase in the percentage of total lipids, which would allow a greater solubilization of the liposoluble oxygen, thus trying to increase the efficiency of the gas exchanges. This would explain the statistically significant increase in total lipid content in the gills of *T. bernacchii* exposed for 10 days to high temperatures.

7.4.3 Fatty acid composition

This is to our knowledge the first study ever published on FA composition of total lipids in gills of *T. bernacchii* exposed to high temperatures. The choice of temperatures of 0, +1 and +2 °C as thermal stress is based on shelf water warming up to +0.8-+1.4 °C predicted by ~2200 for the Ross Sea region (Timmermann and Hellmer 2013). A short experimental period was chosen because our aim was to investigate the ability of *T. bernacchii* to rapidly respond to rising temperatures. However, links between short-term temperature tolerance and prospects for surviving changing environments seem to exist (Barnes et al. 2010).

Table 7.3 shows FAs composition of the gill filaments of specimens of *T. bernacchii* from different groups (see Table S7.2 in Supporting Information for fatty acids nomenclature). Only FAs >0.5% are reported, and the complete dataset is reported in Table S7.3. Minor FAs, with a percentage <1% were excluded from any statistical analyses because their concentrations were close to the Limit of Detection. Results highlighted statistically significant differences in the FA composition of the gills of specimens of control group C1 compared to control group C0. In particular, acclimatized control group C1 showed a statistically significant higher percentage of myristic (14:0), palmitoleic (16:1n-7), gondoic (20:1n-9), eicosapentaenoic (EPA, 20:5n-3), and arachidonic (20:4n-6) acids, and of the sum of mono-unsaturated fatty acids (MUFA), and total n-6 Polyunsaturated fatty acids (n-6-PUFA), than control group C0. Moreover, C1 specimens showed a statistically

significant reduction in the percentage of palmitic (16:0), stearic (18:0), and docosahexaenoic (22:6n-3) acids, and of the sum of saturated fatty acids (SFA), compared to control group C0. Then, to study the influence of temperature and exposure time on the composition of fatty acids in the gills, the treated groups were compared with specimens acclimatized in the tank (C1 group). Figure S7.1 in Supporting Information graphically visualizes statistically significant results. Concerning the most important SFAs, myristic acid (14:0) showed a significant decrease in all treated groups compared to control group, whereas palmitic (16:0) and stearic (18:0) acids increased significantly in all treated groups, except 16:0 for specimens exposed to 0 °C for 10 days. Therefore, total SFAs percentages were higher in temperature-treated specimens than in control ones. The most important mono-unsaturated fatty acids (MUFAs) showed in general a significant percentage reduction in treated groups *vs* control one: palmitoleic acid (16:1n-7), significantly reduced at 0 and 1 °C after 1 day, and after 5 days at all tested temperatures, whereas oleic acid 18:1n-9 significantly decreased in all treated groups. *Cis*-vaccenic acid (18:1n-7) showed a significant reduction after 1 day at 0 °C, for all times of exposure to 1°C, and after 5 days to 2 °C. Gondoic acid (20:1n-9) showed a significant percentage reduction in all treated groups, whereas no variations were recorded at 0 °C after 1 day with respect to control group. Erucic acid (22:1n-9) showed a significant decrease only after 5 days in specimens exposed to 1 °C. As a result of these variations, total MUFAs showed a significant reduction with respect to control group for all tested temperatures and times of exposure. The EFA eicosapentaenoic acid (EPA, 20:5n-3) showed values significantly higher than the control group for specimens exposed to 0 °C for 1 and 10 days, and to 1 °C for all exposure times; in specimens exposed to 2 °C for 1 and 10 days, EPA percentage showed instead values significantly lower than the control group. The other important EFA, docosahexaenoic acid (DHA, 22:6n-3) showed a similar behavior: a significant percentage increase was recorded in specimens exposed to 0 and 1 °C for all exposure times, whereas a significant decrease was recorded in specimens exposed to 2 °C for 1 day. Total omega 3 PUFA showed a statistically significant increase at 0 and 1 °C for all exposure times, and a statistically significant decrease in specimens exposed to 2 °C for 1 and 10 days, with respect to control group C1. Total n-6-PUFA showed a percentage statistically higher in specimens exposed to 0 °C for 1 day and at 1 °C for 5 and 10 days, and a percentage statistically lower in specimens exposed to 2 °C for 10 days, than control group. Overall, PUFAs, represented for about 80% by the sum of EPA and DHA, showed the same trend of n-3-PUFA.

7.4.4 Principal Component Analysis

To better understand the relationships between FAs composition and exposure to different temperatures for different times, a multivariate analysis (Principal Component Analysis, PCA) was performed to reduce the dimensionality of the data set to few components that summarize the information contained in the overall data set. The percentage of total lipids (*vs* dw, %TL) were included in the data-set, together with FAs >1%. As already pointed out, lipidic profile of the group C1 presented statistically significant differences compared to the group C0, highlighting how, even if specimens of the two control groups were exposed to the same temperature, the different environment (aquarium *vs* sea) led to statistically significant variations in the composition of fatty acids in the gill tissue. Consequently, to study the influence of high temperatures and exposure time, the treated groups were compared only with the acclimatized fish in the aquarium (group C1), and for this reason, the C0 group was excluded from the PCA. By applying Principal Component Analysis to the data set (10 observations, 12 variables), it was possible to extract four significant, cross-validated principal components, that accounted for 86.89% of the variability in the original data (Table 7.4). On examining the loading matrix (Table 7.4) and the graphical distribution of analyzed groups on the reported biplots of PC1 *vs* PC2 (Fig. 7.3a), and PC1 *vs* PC3 (Fig. 7.3b), specimens were divided on the basis of their FAs composition. In the biplot reported in Fig. 7.3a (PC1 vs PC2), showing *loadings* and *scores* plots simultaneously, specimens were generally spatially separated on the basis of temperature of exposure: control group, exposed to -1.8 °C in the upper-right quadrant, specimens exposed to 0 °C in the upper left quadrant, specimens exposed to 1 °C in the lower left quadrant, and specimens exposed to 2 °C in the lower right quadrant. Control group C1 (positive scores on PC1 and PC2) was characterized by higher percentages of 14:0, 16:1n7, 18:1n-7 and 18:1n-9 (positive loadings on PC1), 20:1n-9, 22:1n-9 (positive loadings on PC2), and lower percentages of 20:5n-3, 22:6n-3 (negative loadings on PC1), 16:0 and 18:0 (negative loadings on PC2) than other groups. Specimens exposed to 0 °C for 1 and 10 days (negative scores on PC1 and positive scores on PC2) showed generally higher percentages of 20:5n-3, 22:6n-3, 20:1n-9, 22:1n-9, 18:2n-6, and lower percentages of 14:0, 16:1n-7, 18:1n-7 and 18:1n-9, and 16:0 and 18:0 than other groups. Specimens exposed to 1 °C (negative scores on PC1 and PC2), presented higher percentages of 20:5n-3, 22:6n-3, and 16:0 and 18:0, and lower percentages of 14:0, 16:1n-7, 18:1n-7 and 18:1n-9, and 20:1n-9, 22:1n-9, 18:2n-6 than other groups. Finally, specimens exposed to

2 °C (positive score on PC1 and negative scores on PC2) were characterized by higher percentages of 14:0, 16:1n-7, 18:1n-7, 18:1n-9, and (except for 2°C-5 days) 16:0 and 18:0, and lower percentages of 20:5n-3, 22:6n-3, 20:1n-9, and 22:1n-9, than other groups. On the basis of these results we can note that PC1 and PC2 were dominated by the temperature, that explained ~60% of the variance.

PC3 (Fig. 7.3b) was mainly dominated by the percentage of total lipids: specimens exposed to high temperatures for 10 days (positive scores), were characterized by higher percentages of total lipids and 16:1n-7, and lower percentages of 18:0, and 18:2n-6, than corresponding groups exposed to the same temperatures for 1 and 5 days (negative scores). PC3 was then associated with the time of exposure. Finally, PC4 is dominated by the contrast between 16:1n-7, 18:2n-6 (positive loadings) and 18:0, 20:1n-9 and 22:1n-9 (negative loadings) (Table 7.4), but did not provide further information about FA composition differences between studied groups.

7.4.5 Comparison with muscle tissue

In the same specimens of *T. bernacchii* analyzed in this study, no differences in FA composition of total lipids between specimens sacrificed as captured (C0 group) and specimens acclimated in tank for 10+10 days (C1 group) were evidenced in the muscle tissue (Truzzi et al. 2018) whereas in gill tissue statistically significant differences were highlighted between the two control groups. This difference between gill and muscle response to high temperatures is plausible given the functional differences between the two tissues types. Such hypothesis is supported also by PCA results, that demonstrated that the thermal shock influenced tissues in a different manner: for the muscle tissue, the exposition time to high temperatures is the most relevant factor, considering that it is associated to PC1, and explained 47% of the variance; temperature, associated to PC2, is responsible only for 26%. In the case of gill tissue, a relevant effect of temperature was highlighted for FA composition: PC1 and PC2 were associated to the temperature of exposition, explaining 60% of the variance, whereas exposition time associated to PC3, explained only 18% of the variance, and it is mainly laid to changes in total lipid content.

From the results of this study, we found that high temperatures caused not only a variation in total lipid content but also a significant change in FAs composition of lipids in gill tissue. Different explanations can be hypotized to explain these changes.

7.4.6 Lipid peroxidation

Several studies showed that the exposition of *T. bernacchii* to high temperatures did not cause a statistically significant variation of the anti-oxidant system or oxidative damage such as lipid peroxidation in the gill tissue (Klein et al. 2017; Machado et al. 2014; Souza et al. 2018). Wilhelm-Filho (1994) demonstrated the ability of fishes to excrete hydrogen peroxide *via* gills, thus protecting tissue from oxidative damage. Moreover, other studies showed that thermal shock increases lipid peroxidation in gills (Parihar et al., 1997, Forgati et al. 2017, Klein et al. 2017). In light of these evidences, we can not exclude an alteration of FA composition due to lipid peroxidation, that can act particularly on MUFA and PUFA, more susceptible to the oxidation than SFA.

7.4.7 Metabolic pathways of FA

We can consider changes in FA composition taking into account the metabolic pathways of FA, considering that in *T. bernacchi* an activity of enzymes involved in FA biosynthesis has been demonstrated: Porta et al. (2013) found and characterized Δ9-desaturase in *Trematomus bernacchii*, and demonstrated that this gene is expressed. Moreover, Huth and Place (2016) demonstrated an up- and down-regulation of genes involved in lipid metabolism in specimens of *Trematomus bernacchii* exposed to multiple stressors (increased temperature and pCO_2), or an induction of the expression of many genes associated with lipid biosynthesis. The gill tissue of *T. bernacchii* is characterized by high levels of MUFA (~56%), as reported in other studies for the Antarctic fish *Pleuragramma antarcticum* (Mayzaud et al. 2011) or for sub-Artic fishes (Murzina et al. 2013). The PC2 demonstrated a contrast between specimens exposed to lower temperature, -1.8 and 0 °C (positive scores), characterized by higher levels of 18:1n-9, 20:1n-9 and 22:1n-9 and lower levels of 16:0 and 18:0 than specimens exposed to higher temperatures, +1 and +2 °C (negative scores). These MUFA cited above are metabolites of palmitic and stearic acids: it

seems that specimens exposed to higher temperatures responded to a thermal schock reducing the metabolic pathways of 16:0 and 18:0, and leading then to an increase of them and to a reduction of their metabolites (Table 7.3). This behavior is evident already after 1 day of exposition at 2 °C, and after 5 days at 1 °C (Fig. 7.3a). Moreover, PC1 showed a contrast between 18:1n-9, 18:1n-7 (positive coefficients) and 22:6n-3 and 20:5n-3 (negative coefficient). Specimens exposed to lower temperatures (0 and 1 °C) from 1 to 10 days showed higher percentages of 22:6n-3 and 20:5n-3 than control group. The high percentage of EPA (20:5n-3) found in lipids of gill tissue is consistent with literature data, where Antarctic fish species had significantly higher levels of EPA and lower levels of arachidonic acid 20:4n-6 than non-Antarctic species (Malekar et al. 2018). In specimens exposed to 2 °C, FA changes are different from those in specimens exposed to 0 or 1 °C: high levels of MUFA were maintained, whereas specimens showed lower PUFA- and higher SFA-percentage than the control group and than specimens exposed to 0 and 1 °C. Based on these results, we suppose a different metabolic response to warm acclimation based on temperature of exposition, as found in other studies, showing indeed that different temperature of exposition determine different physiological modifications in specimens of *T. bernacchii*, or in other species (Hudson et al. 2008; Jayasundara et al. 2013; Mueller 2014; Machado 2014; Sandersfeld et al. 2015; Malekar et al. 2018).

From the literature, it is known that *T. bernacchii* does not respond to a thermal shock with changes in oxygen consumption during acclimation, or an increase of heat-shock proteins, or changes in messenger RNA (Place and Hoffmann 2005, Strobel et al. 2013, Huth and Place 2016). Then, these metabolic changes could be consistent with the need to maintain a proper membrane fluidity.

7.4.8 Homeoviscous response

The monounsaturated fatty acids 16:1n-7, 18:1n-7 and 18:1n-9 are generally associated with membrane fluidity (Hazel 1983; Schulte 2007; Tiku et al. 1996). The presence of high levels of MUFA in membrane cell of fishes adapted to live in a cold environment reflects the ability to maintain the proper fluidity of biological membranes over a certain range (Sinensky 1974). With increasing temperature, (follow the arrow in Fig. 7.3a), fish tried to reduce MUFA levels, as highlighted above, and this change could be consistent with the attempt to maintain membrane functionality, taking into account its important osmo-

regulatory function in the gill tissue. The negative correlation between the content of unsaturated fatty acids and environmental temperature has been confirmed in many species of fish, both freshwater and marine (Ackman 1989).

In this study, recorded EPA and DHA values were significantly higher in specimens exposed to 0 and 1 °C than those in the control group. EPA and DHA are important components of biological cell membranes and their increase could indicate the need to regulate membrane fluidity modifying the ratio between MUFA and PUFA (Brodte et al. 2008). Specimens exposed to 2 °C showed a different behavior, with 20:5n-3 and 22:6n-3 decreasing or without modification compared to control group. Probably, at 2 °C, *T. bernacchii* needs a different change in the unsaturation degree compared to animals exposed to lower temperatures. DHA seems to have a structural advantage over EPA in contributing to membrane fluidity due to the expanded molecular conformation (Hashimoto et al. 2006). These important omega 3 have also other physiological roles, such as anti-inflammatory and anti-thrombotic activity, modulation of the functionality of visual and neuronal processes (Palacios-Pelaez et al. 2010; Serhan et al. 2008), so their role in a warm-acclimation response of *T. bernacchii* need further investigation.

7.4.9 Pathological changes or acclimation response?

Numerous studies on warm acclimation of *T. bernacchii* indeed showed that gill tissue is able to respond to high temperature with an increased Na^+/K^+-ATPase activity and a hypo-osmoregulation (Brauer et al. 2005; Gonzalez-Cabrera et al. 1995; Guynn et al. 2002; Morrison et al. 2006), or with an induction of the expression of many genes associated with lipid biosynthesis (Buckley and Somero 2009). These results presuppose that the functionality of the membrane is maintained. Our results on a likely change on FA metabolism, togheter with an increase of total lipids to increase oxygen solubilization following a major oxygen demand and a contextually decrease in oxygen solubility at high temperatures (Abele and Puntarulo 2004; Pörtner 2001), indicated that *T. bernacchii* exposed to thermal shock tries to acclimate to rising temperatures implementing changes aimed at maintaining the functionality of the gill tissue. Moreover, in this kind of experiment, where *T. bernacchii* was exposed to temperatures up to 4 °C, the animal was capable of living for several weeks without showing signs of suffering (Podrabsky and Somero 2006,

Bilyk and DeVries 2011). Therefore, we can hypothize that FA changes directly reflect a beneficial shift towards an acclimation response, rather than pathological changes.

7.5 Conclusions

To the best of our knowledge, no data are so far available for total lipid percentage and FA composition in gills of *T. bernacchii*. Microwave-assisted extraction of lipids from a lyophilized sample of gill tissue is an environmental friendly (solvent-sparing) technique that reduces the extraction time and gives results consistent with the official method of Bligh and Dyer. In this study, we demonstrated that *T. bernacchii* responds to a thermal shock modifying FA composition and lipid content of gills, implementing changes aimed at maintaining the functionality of the tissue. The Principal Component Analysis helped us to hypothesize that the thermal shock affects the composition of FAs in the gills mainly through an alteration of the metabolic pathways of FAs, and that these changes in lipid metabolism are consistent with the "homeoviscous response". Changes in FA composition depend on the temperature of thermal shock, whereas the time of exposition influences above all the content of total lipids. In light of previous evidences, and of this study, we suppose that *T. bernacchii* is able to rapidly acclimate to a thermal shock. After more than 50 years of research focused on the adaptation of Antarctic notothenioids to climate warming, there are still many questions that remain to be answered. This study may help to gather important information on the development of the general theory of adaptations of fishes to environmental factors, in particular temperature.

7.6 Acknowledgements

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7.7 Figures, Tables and Supplementary materials

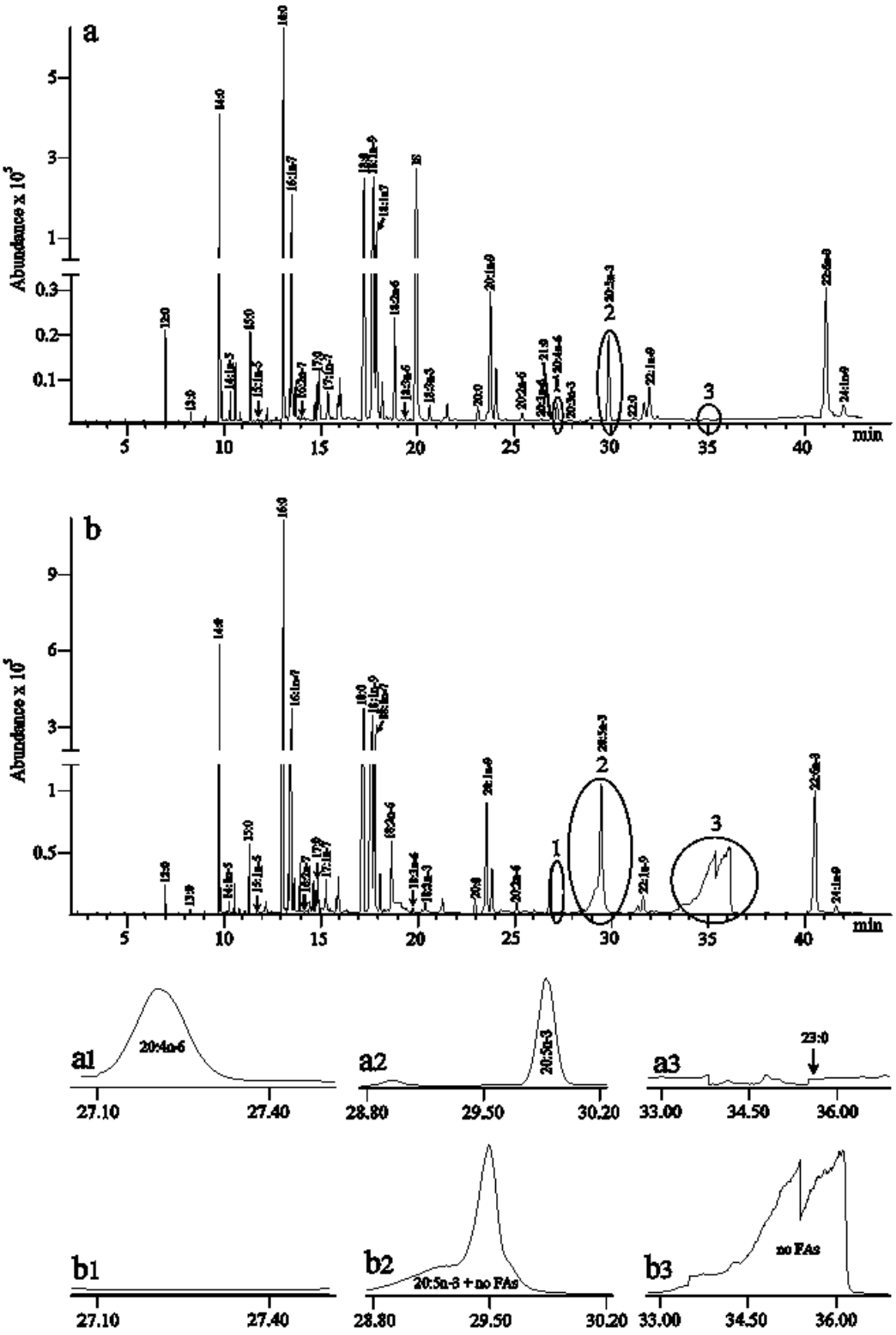


Figure 7.1. Example of chromatograms obtained from total lipid fraction of gills of *T. bernacchii* extracted with MAE (a) or Blig & Dyer (b) methods.

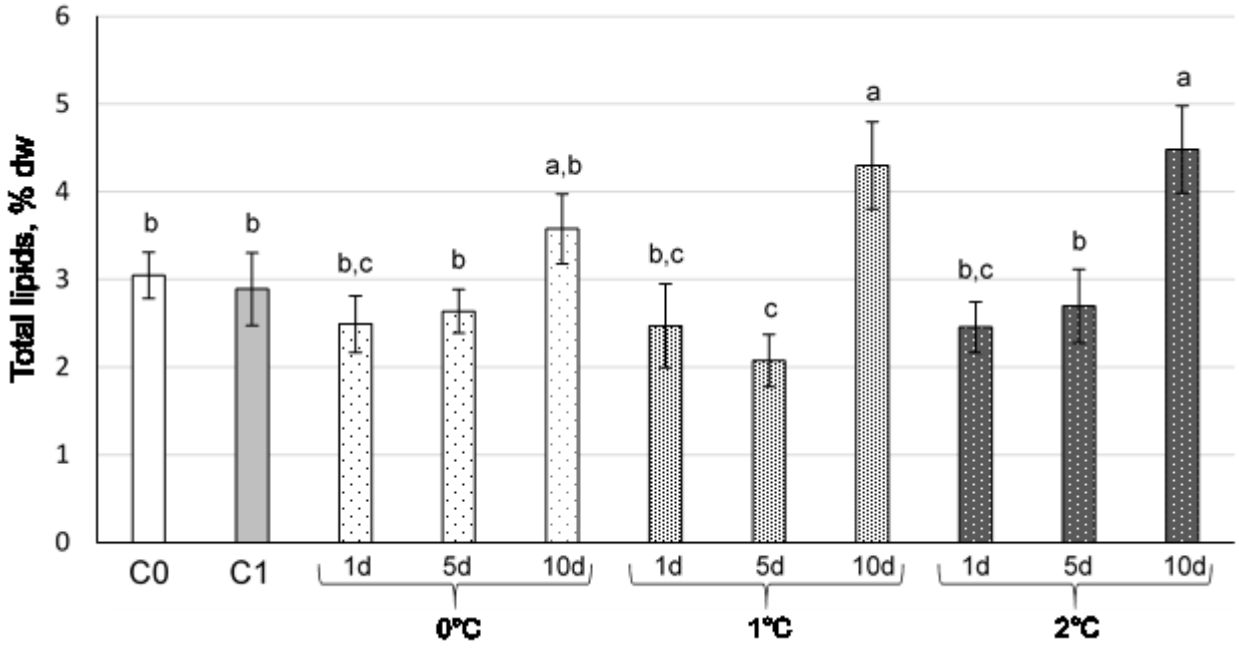


Figure 7.2. Total lipids (as % dw) of gills of specimens of *T. bernacchii* divided *per* group. C0, control fishes as captured; C1, control fishes after acclimation; 1d, 5d and 10d indicate the time of exposure (1,5, and 10 days respectively) at the temperature indicated of 0, 1 or 2 C. Error bars represent standard deviation.

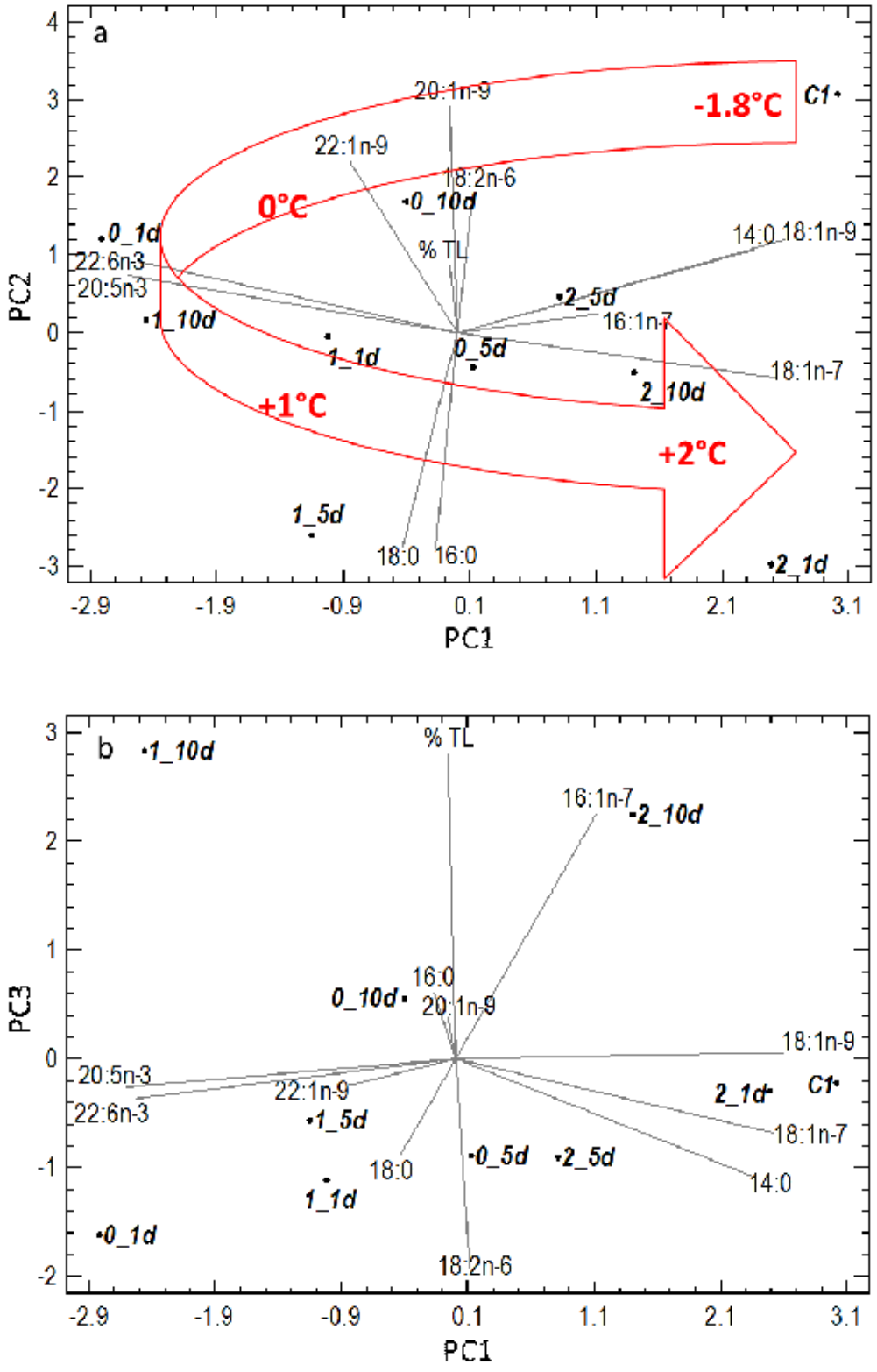


Figure 7.3. 2D biplot of PC1 *vs* PC2 (a) and PC1 *vs* PC3 (b). C1, control fishes after acclimation. Other labels indicate groups exposed to the temperature indicated (0, 1 or 2 °C), followed by the time of exposure; for example: 0_1d, 0_5d and 0_10d indicate animals exposed at 0 C for 1, 5, and 10 days, respectively.

Table 7.1.Percentage of lipids extracted from the gills of *T. bernacchii*: comparison between extraction methods.

n° sample	% lipid ww		
	MAE + lyophilization step	MAE without lyophilization step	Bligh & Dyer
1	0.4±0.3	0.1±0.1	1.0 ± 0.2
2	0.5±0.2	0.6±0.3	0.5 ± 0.1
3	0.9±0.4	0.3±0.2	0.6 ± 0.3
4	0.6±0.2	0.2±0.1	0.9 ± 0.2
5	0.3±0.1	0.4±0.2	0.7 ± 0.3
6	1.0±0.3	0.1±0.1	1.2 ± 0.4
Mean±SD	0.6±0.3	0.3±0.2	0.8 ± 0.3

Table 7.2 Lipid percentage in the gills of *T. bernacchii* and comparison with published data about Antarctic notothenioid fishes.

Reference	Site, Antarctica	specie	Sampling time	% Lipids, ww	% Lipids, dw	Extraction method
This work	Terra Nova Bay, Ross Sea	*T. bernacchii*	Austral Summer, 2014-2015	0.6 ± 0.3 (min-max)	3.2 ± 1.1 (min-max)	MAE
				0.8 ± 0.3 (min-max)	4.2 ± 1.2 (min-max)	Bligh and Dyer
Ríos et al. 2017	King George Island/Isla 25 de Mayo	*T. newnesi*	Austral Summer, 2008-2011		5.15 ± 3.7 (1.24-15.94)	Soxhlet
		Notothenia Rossii			2.14 ± 0.6 (1.64-3.31)	Soxhlet
Lana et al. 2014	King George Island/Isla 25 de Mayo	*Notothenia coriceps*	Austral Summer, 2008-2011		3.05 ± 0.15	Soxhlet

Table 7.3 Composition of major FAs (% *vs* total FAs, mean±SD) of gill tissue of *T. bernacchii* held at different temperatures for different exposure times. Means within rows bearing different letters are significantly different ($p<0.05$).

[+]includes 12:0, 13:0, 15:0, 20:0, 21:0, 22:0, 23:0, 24:0

FAs	C0	C1	1day			5 days			10 days		
			0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C
14:0	6.5±0.2^{c}	7.6±0.3^{a}	6.0±0.3^{d}	6.8±0.6^{b,c}	6.7±0.2^{b,c}	7.0±0.3^{b}	6.2±0.3^{d}	6.7±0.2^{b,c}	6.6±0.2^{c}	5.7±0.2^{e}	6.5±0.3^{c}
16:0	13.2±0.6^{b,c}	11.6±0.5^{a}	12.8±0.4^{b}	13.6±0.7^{c}	14.2±0.4^{c,d}	13.4±0.8^{b,c}	13.4±0.4^{b,c}	12.6±0.4^{b}	12.1±0.4^{a,b}	13.3±0.4^{b,c}	14.1±0.5^{c,d}
18:0	8.3±0.4^{e}	3.9±0.2^{a}	6.9±0.4^{c,d}	5.8±0.4^{b}	8.0±0.4^{e}	7.0±0.7^{c,d}	9.2±0.7^{f}	7.5±0.3^{d}	5.6±0.5^{b}	5.5±0.4^{b}	6.6±0.3^{c}
Total SFAs[+]	29.1±0.6^{d,e}	24.3±0.6^{a}	27.1±0.9^{c}	27.5±0.7^{c}	30.1±0.7^{e}	28.5±0.6^{d}	30.1±0.9^{e}	28.0±0.6^{c,d}	25.4±0.7^{b}	25.5±0.6^{b}	28.4±0.7^{d}
16:1n-7	11.2±0.5^{d,e}	13.6±0.4^{b}	10.5±0.5^{e}	12.7±0.5^{c}	13.6±0.5^{b}	11.8±0.5^{d}	11.6±0.6^{d}	12.1±0.4^{c,d}	13.3±0.4^{b,c}	14.5±0.5^{a}	13.2±0.4^{b,c}
17:1n-7	0.65±0.06	0.94±0.05	0.92±0.03	1.19±0.06	0.79±0.07	0.79±0.03	0.87±0.05	0.82±0.12	0.89±0.05	0.82±0.09	1.07±0.07
18:1n-9	26.7±0.7^{a}	26.9±0.7^{a}	21.9±0.5^{d,e}	21.7±0.6^{d,e}	23.7±0.7^{c}	23.0±0.9^{c,d}	22.4±0.6^{d}	24.9±0.5^{b}	23.3±0.8^{c,d}	21.4±0.5^{e}	24.8±0.5^{b}
18:1n-7	6.5±0.2^{b,c}	6.8±0.2^{b}	6.2±0.2^{c}	6.3±0.2^{c}	7.4±0.2^{a}	6.5±0.2^{b,c}	6.1±0.3^{c}	6.4±0.2^{c}	6.5±0.3^{b,c}	5.6±0.2^{d}	6.7±0.3^{b}
20:1n-9	3.9±0.2^{d}	5.4±0.1^{a}	5.1±0.3^{a,b}	4.4±0.1^{c}	4.0±0.3^{d}	4.8±0.3^{b}	4.4±0.3^{b,c}	5.0±0.2^{b}	4.9±0.2^{b}	4.9±0.2^{b}	4.9±0.2^{b}
22:1n-9	1.5±0.1^{b}	1.4±0.1^{b}	1.7±0.2^{ab}	1.4±0.2^{b,c}	1.2±0.1^{b,c}	1.3±0.1^{b,c}	1.2±0.1^{c}	1.4±0.1^{b}	1.6±0.2^{ab}	1.3±0.1^{b,c}	1.5±0.1^{a,b}
24:1n-9	0.86±0.07	0.57±0.03	0.71±0.08	0.50±0.10	0.57±0.05	0.65±0.07	0.52±0.03	0.61±0.06	0.72±0.02	0.62±0.01	0.66±0.06
Total MUFAs[*]	51.5±0.5^{b}	56.1±0.5^{a}	47.4±0.8^{d}	48.6±0.6^{c}	51.6±0.6^{b,c}	49.3±0.8^{c}	47.5±0.6^{d}	51.6±0.5^{b,c}	51.7±0.9^{b,c}	49.5±0.9^{c}	53.2±0.5^{b}
18:3n-3	0.39±0.02	0.54±0.06	0.64±0.04	0.74±0.09	0.52±0.02	0.51±0.12	0.52±0.02	0.70±0.04	0.64±0.05	0.70±0.01	0.47±0.02
20:2n-6	0.39±0.06	0.67±0.06	0.80±0.13	0.63±0.20	0.99±0.01	0.77±0.28	0.84±0.12	0.63±0.04	0.77±0.07	1.03±0.04	0.55±0.01
20:5n-3	6.1±0.8^{f}	9.1±0.2^{c}	11.3±0.2^{a}	10.8±0.3^{a,b}	8.6±0.2^{d}	8.9±0.6^{c,d}	10.5±0.2^{b}	9.5±0.2^{c}	10.7±0.6^{a,b}	11.2±0.3^{a}	8.3±0.2^{e}
22:6n-3	9.7±0.7^{f}	5.6±0.4^{b}	8.8±0.3^{f}	7.9±0.3^{e}	4.5±0.3^{a}	8.3±0.3^{e,f}	6.5±0.3^{c}	5.7±0.4^{b}	7.2±0.3^{d}	8.1±0.3^{e}	5.7±0.5^{b}
Total n-3 PUFAs°	16.1±0.9^{a,b}	15.3±0.3^{b}	20.7±0.6^{e}	19.5±0.2^{d}	13.8±0.2^{a}	17.8±0.4^{c}	17.6±0.4^{c}	15.9±0.2^{b}	18.6±0.6^{c,d}	20.2±0.5^{e}	14.5±0.2^{a}
18:2n-6	1.7±0.1^{b,c}	1.8±0.1^{a,b}	1.8±0.2^{a,b}	1.9±0.1^{a}	1.7±0.2^{a,b}	1.7±0.1^{b,c}	1.5 ±0.2^{c}	1.8±0.1^{a,b}	1.7±0.1^{a,b}	1.6±0.2^{c}	1.5±0.2^{c}
20:4n-6	0.9±0.1^{e}	1.4±0.1^{c}	1.7±0.2^{a,b}	1.3±0.2^{c,d}	1.3±0.2^{c,d}	1.5±0.1^{b,c}	2.0±0.2^{a}	1.5±0.1^{b,c}	1.1±0.1^{d}	1.6±0.1^{b}	1.4±0.3^{b,c}
Total n-6 PUFAs^	3.2±0.2^{d}	4.2±0.2^{b}	4.8±0.1^{a}	4.3±0.2^{b}	4.3±0.1^{b}	4.4±0.3^{a,b}	4.8±0.3^{a}	4.4±0.2^{a,b}	4.3±0.2^{b}	4.8±0.3^{a}	3.8±0.2^{c}
Total PUFAs°^	19.4±0.9^{a,b}	19.6±0.4^{b}	25.6±0.6^{e}	23.9±0.3^{d}	18.2±0.4^{a}	22.2±0.5^{c}	22.5±0.5^{c}	20.3±0.3^{b}	22.9±0.7^{c,d}	25.0±0.6^{e}	18.4±0.3^{a}

[*]includes 14:1n-5, 15:1n-5
°includes 20:3n-3
^includes 18:3n-6, 20:3n-6

Table 7.4 Principal Component Analysis. Eigenvalues, explained and cumulative variance, loadings of the variables for the first three PCs.

	Principal Components			
	1	2	3	4
Variance explained				
Eigenvalues	3.823	3.343	2.152	1.104
% of variance	31.90	27.86	17.93	9.196
Cumulative %	31.90	59.76	77.69	86.89
Factor loadings				
14:0	0.404	0.178	-0.243	0.170
16:0	-0.029	-0.461	0.138	0.029
18:0	-0.074	-0.457	-0.199	-0.365
16:1n-7	0.191	0.041	0.506	0.505
18:1n-9	0.445	0.200	0.012	-0.251
18:1n-7	0.433	-0.096	-0.154	-0.016
20:1n-9	-0.010	0.482	0.086	-0.343
22:1n-9	-0.145	0.365	-0.054	-0.366
18:2n-6	0.020	0.267	-0.433	0.442
20:5n-3	-0.447	0.122	-0.057	0.227
22:6n-3	-0.432	0.152	-0.081	0.088
%TL	-0.010	0.145	0.629	-0.110

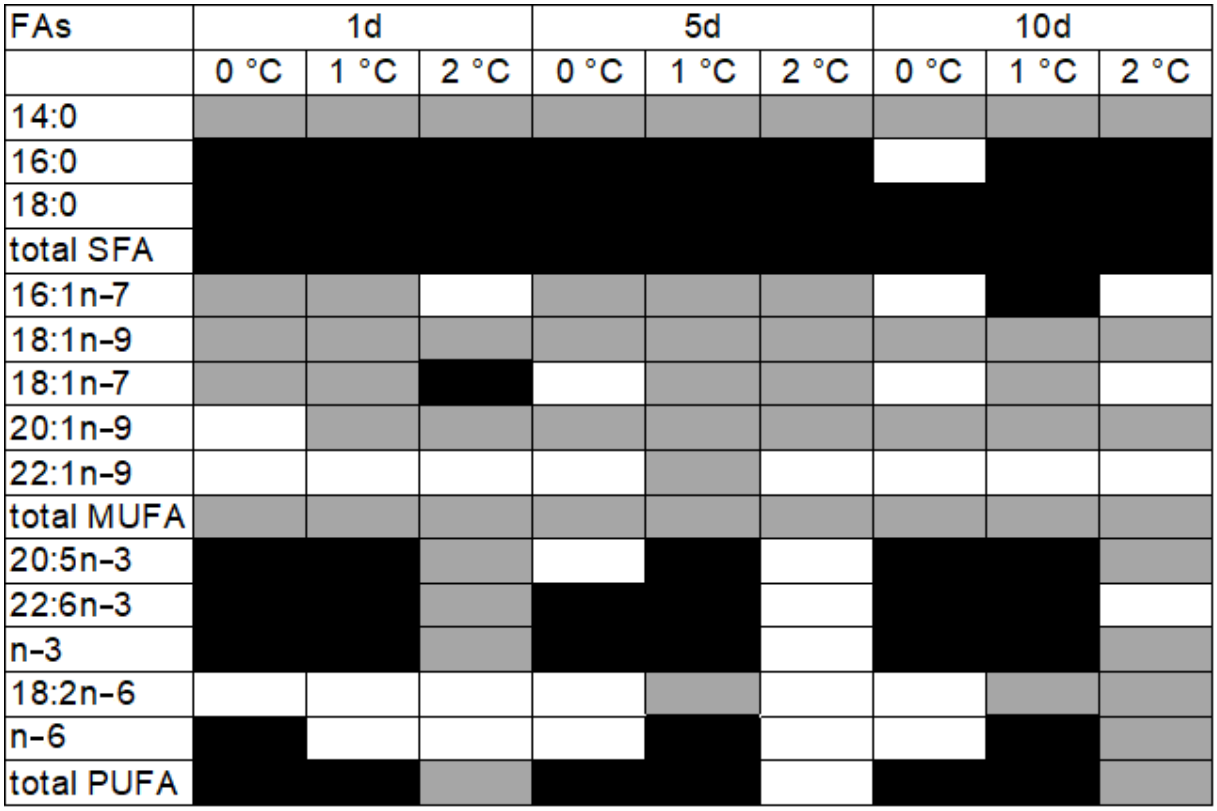
Table S7.1. Nomenclature of fatty acids cited in the text.

FA	IUPAC name	Other names
12:0	Dodecanoic acid	Lauric acid
13:0	Tridecanoic acid	Tridecylic acid
14:0	Tetradecanoic acid	Myristic acid
14:1n-5	(9Z)-Tetradec-9-enoic acid	Myristoleic acid
15:0	Pentadecanoic acid	
15:1n-5	(10Z)-Pentadec-10-enoic acid	Cis-10-Pentadecenoic acid
16:0	Hexadecanoic acid	Palmitic acid
16:1n-7	(9Z)-Hexadec-9-enoic acid	Palmitoleic acid
17:0	Heptadecanoic acid	Margaric acid
17:1n-7	(10Z)-Heptadec-10-enoic acid	Cis-10-heptadecenoic acid
18:0	Octadecanoic acid	Stearic acid
18:1n-9	(9Z)-Octadec-9-enoic acid	Oleic acid
18:2n-6c	(9Z,12Z)-9,12-Octadecadienoic acid	Linoleic acid
18:2n-6t	(9E,12E)-Octadeca-9,12)-dienoic acid	Linolelaidic acid
18:3n-6	(6Z,9Z,12Z)-Octadeca-6,9,12-trienoic acid	γ-Linolenic acid
18:3n-3	(9Z,12Z,15Z)-Octadeca-9,12,15-trienoic acid	α-Linolenic acid
20:0	Eicosanoic acid	Arachic acid
20:1n-9	(11Z)-Icosa-11-enoic acid	Gondoic acid
20:2n-6	(11Z,14Z)-Icosa-11,14-dienoic acid	Eicosadienoic acid
20:3n-6	(8Z,11Z,14Z)-Icosa-8,11,14-trienoic acid	Dihomo-γ-linolenic acid
21:0	Heneicosanoic acid	Henicosanoic acid
20:4n-6	(5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoic acid	Arachidonic acid
20:3n-3	(11Z,14Z,17Z)-Icosa-11,14,17-trienoic acid	Eicosatrienoic acid
20:5n-3	(5Z,8Z,11Z,14Z,17Z)-Icosa-5,8,11,14,17-pentaenoic acid	Eicosapentaenoic acid
22:0	Docosanoic acid	Behenic acid
22:1n-9	(13Z)-Docos-13-enoic acid	Erucic acid.
23:0	Tricosanoic acid	Tricosylic acid
24:0	Tetracosanoic acid	Lignoceric acid
22:6n-3	(4Z,7Z,10Z,13Z,16Z,19Z)-Docosa-4,7,10,13,16,19-hexaenoic acid	Docosahexaenoic acid
24:1n-9	(15Z)-Tetracos-15-enoic acid	Nervonic acid

Table S7.2. FAs composition (% *vs* total FAs, mean±SD) of the gills tissue of *T. bernacchii* held at different temperatures for different time.

FAs	C0	C1	1day			5 days			10 days		
			0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C	0 °C	+1 °C	+2 °C
12:0	0.28±0.01	0.18±0.02	0.25±0.05	0.20±0.01	0.18±0.02	0.19±0.02	0.18±0.01	0.25±0.03	0.22±0.02	0.15±0.03	0.19±0.02
13:0	0.03±0.01	0.04±0.01	0.03±0.01	0.05±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.04±0.01	0.03±0.01	0.03±0.01	0.03±0.01
14:0	6.5±0.2^{c}	7.6±0.3^{a}	6.0±0.3^{d}	6.8±0.6^{b,c}	6.7±0.2^{b,c}	7.0±0.3^{b}	6.2±0.3^{d}	6.7±0.2^{b,c}	6.6±0.2^{c}	5.7±0.2^{e}	6.5±0.3^{c}
15:0	0.34±0.01	0.50±0.03	0.40±0.02	0.53±0.03	0.46±0.01	0.38±0.02	0.43±0.03	0.44±0.03	0.43±0.03	0.45±0.03	0.53±0.02
16:0	13.2±0.6^{b,c}	11.6±0.5^{a}	12.8±0.4^{b}	13.6±0.7^{c}	14.2±0.4^{c,d}	13.4±0.8^{b,c}	13.4±0.4^{b,c}	12.6±0.4^{b}	12.1±0.4^{a,b}	13.3±0.4^{b,c}	14.1±0.5^{c,d}
17:0	0.27±0.01	0.24±0.02	0.33±0.03	0.34±0.03	0.30±0.03	0.27±0.02	0.34±0.01	0.27±0.01	0.27±0.01	0.27±0.01	0.35±0.03
18:0	8.3±0.4^{e}	3.9±0.2^{a}	6.9±0.4^{c,d}	5.8±0.4^{b}	8.0±0.4^{e}	7.0±0.7^{c,d}	9.2±0.7^{f}	7.5±0.3^{d}	5.6±0.5^{b}	5.5±0.4^{b}	6.6±0.3^{c}
20:0	0.17±0.01	0.17±0.01	0.23±0.03	0.15±0.02	0.17±0.02	0.19±0.02	0.24±0.01	0.19±0.01	0.14±0.01	0.15±0.01	0.17±0.01
21:0	0.05±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.01±0.01	0.01±0.01
22:0	0.07±0.01	0.03±0.01	0.04±0.01	0.03±0.01	0.07±0.02	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01
23:0	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
24:0	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
Total SFAs	29.1±0.6^{d,e}	24.3±0.6^{a}	27.1±0.91^{c}	27.5±0.7^{c}	30.1±0.7^{e}	28.5±0.6^{d}	30.1±0.9^{e}	28.0±0.6^{c,d}	25.4±0.7^{b}	25.5±0.6^{b}	28.4±0.7^{d}
14:1n-5	0.30±0.01	0.47±0.02	0.26±0.02	0.37±0.02	0.35±0.03	0.34±0.02	0.28±0.03	0.31±0.02	0.34±0.03	0.32±0.02	0.32±0.03
15:1n-5	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.01±0.01	0.03±0.01
16:1n-7	11.2±0.5^{d,e}	13.6±0.4^{b}	10.5±0.5^{e}	12.7±0.5^{c}	13.6±0.5^{b}	11.8±0.5^{d}	11.6±0.6^{d}	12.1±0.4^{c,d}	13.3±0.4^{b,c}	14.5±0.5^{a}	13.2±0.4^{b,c}
17:1n-7	0.65±0.06	0.94±0.05	0.92±0.03	1.19±0.06	0.79±0.07	0.79±0.03	0.87±0.05	0.82±0.12	0.89±0.05	0.82±0.09	1.07±0.07
18:1n-9	26.7±0.7^{a}	26.9±0.7^{a}	21.9±0.5^{d,e}	21.7±0.6^{d,e}	23.7±0.7^{c}	23.0±0.9^{c,d}	22.4±0.6^{d}	24.9±0.5^{b}	23.3±0.85^{c,d}	21.4±0.5^{e}	24.8±0.5^{b}
18:1n-7	6.5±0.2^{b,c}	6.8±0.2^{b}	6.2±0.2^{c}	6.3±0.2^{c}	7.4±0.2^{a}	6.5±0.2^{b,c}	6.1±0.3^{c}	6.4±0.2^{c}	6.5±0.3^{b,c}	5.6±0.2^{d}	6.7±0.3^{b}
20:1n-9	3.9±0.2^{d}	5.4±0.1^{a}	5.1±0.3^{a,b}	4.4±0.1^{c}	4.0±0.3^{d}	4.8±0.3^{b}	4.4±0.3^{b,c}	5.0±0.2^{b}	4.9±0.2^{b}	4.9±0.2^{b}	4.9±0.2^{b}
22:1n-9	1.5±0.1^{b}	1.4±0.1^{b}	1.7±0.2^{ab}	1.4±0.2^{b,c}	1.2±0.1^{b,c}	1.3±0.1^{b,c}	1.2±0.1^{c}	1.4±0.1^{b}	1.6±0.2^{ab}	1.3±0.1^{b,c}	1.5±0.1^{a,b}
24:1n-9	0.86±0.07	0.57±0.03	0.71±0.08	0.50±0.10	0.57±0.05	0.65±0.07	0.52±0.03	0.61±0.06	0.72±0.02	0.62±0.01	0.66±0.06
Total MUFAs	51.5±0.5^{b}	56.1±0.5^{a}	47.4±0.8^{d}	48.6±0.6^{c}	51.6±0.6^{b,c}	49.3±0.8^{c}	47.5±0.6^{d}	51.6±0.5^{b,c}	51.7±0.9^{b,c}	49.5±0.9^{c}	53.2±0.5^{b}
18:3n-3	0.39±0.02	0.54±0.06	0.64±0.04	0.74±0.09	0.52±0.02	0.51±0.12	0.52±0.02	0.70±0.04	0.64±0.05	0.70±0.01	0.47±0.02
20:3n-3	0.01±0.01	0.09±0.01	0.03±0.01	0.12±0.02	0.12±0.01	0.08±0.02	0.07±0.01	0.07±0.01	0.09±0.02	0.15±0.02	0.08±0.01
20:5n-3	6.1±0.8^{f}	9.1±0.2^{c}	11.3±0.2^{a}	10.8±0.3^{a,b}	8.6±0.2^{d}	8.9±0.6^{c,d}	10.5±0.2^{b}	9.5±0.2^{c}	10.7±0.6^{a,b}	11.2±0.3^{a}	8.3±0.2^{e}
22:6n-3	9.7±0.7^{f}	5.6±0.4^{b}	8.8±0.3^{f}	7.9±0.3^{e}	4.5±0.3^{a}	8.3±0.3^{e,f}	6.5±0.3^{c}	5.7±0.4^{b}	7.2±0.3^{d}	8.1±0.3^{e}	5.7±0.5^{b}
Total n-3 PUFAs	16.1±0.9^{a,b}	15.3±0.3^{b}	20.7±0.6^{e}	19.5±0.2^{d}	13.8±0.2^{a}	17.8±0.4^{c}	17.6±0.4^{c}	15.9±0.2^{b}	18.6±0.6^{c,d}	20.2±0.5^{e}	14.5±0.2^{a}
18:2n-6	1.7±0.1^{b,c}	1.8±0.1^{a,b}	1.8±0.2^{a,b}	1.9±0.1^{a}	1.7±0.2^{a,b}	1.7±0.1^{b,c}	1.5 ±0.2^{c}	1.8±0.1^{a,b}	1.7±0.1^{a,b}	1.6±0.2^{c}	1.5±0.2^{c}
18:3n-6	0.10±0.01	0.22±0.03	0.27±0.04	0.30±0.06	0.26±0.07	0.20±0.08	0.24±0.02	0. 26±0.03	0.31±0.02	0.33±0.06	0.23±0.01
20:2n-6	0.39±0.06	0.67±0.06	0.80±0.13	0.63±0.09	0.99±0.09	0.77±0.08	0.84±0.09	0.63±0.04	0.77±0.07	1.03±0.04	0.55±0.04
20:3n-6	0.12±0.02	0.18±0.03	0.19±0.02	0.20±0.02	0.15±0.01	0.25±0.02	0.18±0.02	0.20±0.01	0.29±0.03	0.21±0.03	0.15±0.01
20:4n-6	0.9±0.1^{e}	1.4±0.1^{c}	1.7±0.2^{a,b}	1.3±0.2^{c,d}	1.3±0.2^{c,d}	1.5±0.1^{b,c}	2.0±0.2^{a}	1.5±0.1^{b,c}	1.1±0.1^{d}	1.6±0.1^{b}	1.4±0.3^{b,c}
Total n-6 PUFAs	3.2±0.2^{d}	4.2±0.2^{b}	4.8±0.1^{a}	4.3±0.2^{b}	4.3±0.1^{b}	4.4±0.3^{a,b}	4.8±0.3^{a}	4.4±0.2^{a,b}	4.3±0.2^{b}	4.8±0.3^{a}	3.8±0.2^{c}
16:2n-7	0.05±0.01	0.08±0.01	0.07±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.06±0.01	0.08±0.01	0. 06±0.01	0.07±0.01
Total PUFAs	19.4±0.9^{a,b}	19.6±0.4^{b}	25.6±0.6^{e}	23.9±0.3^{d}	18.2±0.4^{a}	22.2±0.5^{c}	22.5±0.5^{c}	20.3±0.3^{b}	22.9±0.7^{c,d}	25.0±0.6^{e}	18.4±0.3^{a}

Figure S7.1. Influence of temperature and exposure times on the percentage of FAs in gills of *T. bernacchii*. Only statistically significant changes ($p<0.05$) with respect to the control group C1 (grey, decrease; black, increase; white no significative change) are reported.



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8 Modelling the influence of time and temperature on the levels of fatty acids in the liver of Antarctic fish *Trematomus bernacchii*.

8.1 Abstract

Antartic fish (*Trematomus bernacchii*) are an ideal group for studying the effect of ocean warming on vital physiological and biochemical mechanisms of adaptation, including changes in the fatty acid composition to higher heat tolerance in the sub-zero waters of the Southern Ocean. Despite the awareness of the impact of ocean warming on marine life, bioclimatic models describing the effect of temperature and time on fatty acids levels in marine species have not been considered yet. Changes in the concentrations of fatty acids in liver from *Trematomus bernacchii* were observed after varying simultaneously and systematically the temperature and time. The observed changes were graphically visualized by expressing the fatty acid concentration as a function of the temperature and time using polynomial models.

The fatty acid profiles were determined by gas chromatography prior to acclimation (-1.8°C) and after acclimation (0.0, 1.0 and 2.0 °C) at different times (1, 5 and 10 days). Major changes in fatty acid composition were observed at 1 day of exposition at all temperatures. At 5 days, the fish start adapting to the new temperature condition. The concentrations of saturated fatty acids were almost constant throughout the various conditions. The monounsaturated fatty acids (in particular 18:1n-9) decrease at 1 day for all temperatures. In contrast, the polyunsaturated fatty acids (in particular 20:5n-3 and 22:6n-3) increase throughout the various conditions. The proposed models were in agreement with reported studies on polar and temperate fish, indicating possibly similar adaptation mechanisms for teleost to cope with global warming.

8.2 Introduction

Local climate models are representations of the likely effect of anthropogenic sources on climate (Intergovernmental Panel for Global Climate Change (IPCC) 2000). Bioclimatic models have predicted the extinction of Antarctic toothfish in 30 years under strong warming conditions (Cheung et al. 2008). Data from Pleistocene glaciation has indicated that species more often responded to climate change by local adaptation (Parmesan et al. 2000). It has been reported that gradual increase in water temperature from global warming may result in changes in species composition and acclimation to higher heat tolerance (O'Connor et al. 2007; Cheung et al. 2008). It is undeniable, that sea temperature is the principal factor determining distributions, abundance and physiology of fish species (Pörtner 2001; Roessig et al. 2004). The Commission for the Conservation of the Antarctic Marine Living Resources has pointed out that the Antarctic region and more specifically the Ross Sea have not been dramatically affected by commercial fisheries, whaling and other human activities when compare to its north counterpart (Antarctic and Southern Ocean Coalition (ASOC) 2009). For this particular reason, the Antarctic provides a unique potential for documenting the biological and chemical effects of global warming on a polar marine ecosystem (Antarctic and Southern Ocean Coalition (ASOC) 2009). Fatty acids (FA) are regarded as ideal biological markers of environmental exposure in marine research (Dalsgaard et al. 2003; Könneke and Widdel 2003; Alfaro et al. 2006; Sajjadi and Eghtesadi-Araghi 2011; Copeman et al. 2013; Mourente et al. 2015). The fatty acid levels in liver and muscle and gills of *Trematomus bernacchii* have been recently determined (Truzzi et al. 2017, 2018a, b; Corsolini and Borghesi 2017; Malekar et al. 2018).

Models describing the effect of temperature and time on fatty acids levels in marine species have not been considered in the literature. Some authors have investigated the temporal effect of temperature and tissue type (liver and muscle) on the levels of fatty acids in fish (Copeman et al. 2013). However, the main drawback of this study is that influence of the temperature and time were evaluated at two and five levels, respectively, which precluded the modelling of the factors.

The present research aims at modelling the effect of the variables time and temperature as a function of the levels of fatty acids in liver of Antarctic fish *T. bernacchii*. This ubiquitous specie of stenothermal Antarctic teleost (family

Notothenidae) is well adapted to the extremely low temperatures of the Antarctic region (-1.86°C) and has been regarded as a key indicator for monitoring anthropogenic impact (Regoli et al. 2005; Di Bello et al. 2007, Illuminati et al. 2010).

8.3 Materials and methods

8.3.1 Reagents

Sodium hydroxide, hexane, methanol, boron trifluoride in methanol (20% w v^{-1}) and chloroform were purchased from Merck (Darmstadt, Germany). Cod liver oil standard was from Peter Møller (Lysaker, Norway), fatty acid methyl ester (FAME) pure and model mixture standards were purchased from Nu-Chek Prep (Elysian, MN). The FAME pure standards were 18:1n-7, 20:5n-3, 22:5n-3. The model mixture standards were 2A and 2B 18:1n-7, 20:5n-3, 22:5n-3. The model mixture standards were 2A and 2B (18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6), 3A (18:2n-6, 18:3n-3, 20:4n-6, 22:6n-3), 4A (6:0, 8:0, 10:0, 12:0, 14:0), 6A (16:0, 18:0, 20:0, 22:0, 24:0), 7A (16:1n-7, 18:1n-9, 20:1n-9, 22:1n-9, 24:1n-9), 8A (20:0, 20:1n-9, 20:2n-6, 20:3n-6; 20:4n-6) and 14A (13:0, 15:0, 17:0, 19:0, 21:0). Nonadecanoic acid methyl ester (19:0) internal standard was from Fluka (Buchs, Switzerland). De-ionized water was purified in a Milli-Q system (Milli-Q system Millipore, Milford, MA).

8.3.2 Sampling

The study was performed at the Mario Zuchelli Station at the Terra Nova Bay in the Antarctic region during the Austral summer 2014-2015 (Fig. 8.1). A total of 63 sexually mature Antarctic *T. bernacchii* species (weight 113-503 g, length 32-22 cm) were caught by a fishing rod at depths of 30 m in the location indicated in Fig. 8.1. Three fish were immediately sacrificed (designated as control seawater at 0 day and -1.8 ± 0.2 °C in Table 8.1) and their liver isolated, frozen in liquid N_2 and stored at -80°C. The rest of the fish (60) were transferred and placed into a flow-through circulating seawater tank at -1.8 ± 0.2 °C for acclimation at habitat temperature for 30 days. After the acclimation period, six fish were killed, their liver isolated, frozen in liquid N_2 and stored at -80°C until analysis. The rest of the acclimatized fish (54) were

divided into three groups and transferred to three flow-through circulating seawater tanks at 0.0, +1.0 and +2.0 °C and held at these conditions for 1, 5 and 10 days. Six fish were killed, at each time period, their liver isolated, frozen in liquid N_2 and stored at -80 °C until analysis. The same diet was provided ad libitum once every second day to all fish during the trials (including acclimation period). The diet consisted of chopped cuttlefish (*Sepia officinalis*) and bivalve molluscs (*Adamussium colbecki)*.

8.3.3 Lipid extraction and fatty acids determination

The sampled livers were pooled together (3 livers for the control seawater, 6 for acclimation and 6 for every specific time/temperature condition), minced and homogenized by means of a high dispersing device (Ultra-Turrax T25, Janke e Kunkel, IKA-Labortechnik, Staufen, Germany). The lipids were extracted in duplicate (n=2) by adding 4 ml of chloroform/methanol (2: 1, v v^{-1}) and 0.080 ml of internal standard (19:0 methyl ester) to 200 mg of sample. The extracted lipids were filtered, the residue was discarded and the chloroform/methanol filtrate was evaporated under a stream of nitrogen. The dried residue was saponified according to a method published elsewhere (Torstensen et al. 2004). Briefly, an aliquot (1 ml) of sodium hydroxide in methanol (0.5 M) was added, heated at 100°C for 15 min and cooled in cold water. An aliquot (2 ml) of boron trifluoride in methanol (20%) was added, vortex-mixed, heated at 100°C for 5 min and cooled in cold water. Subsequent aliquots of hexane (2 ml) and water (2 ml) were added, vortex-mixed, centrifuged at 1207×g for 1 min and the hexane phase collected. An additional 2 ml aliquot of hexane is added to the remaining water, vortex-mixed, centrifuged at 1207×g for 1 min and the hexane phase pooled together with the initial collection. Depending on the fat content the sample is either concentrated under nitrogen or diluted with hexane and subsequently subjected to gas chromatography (GC) analysis.

8.3.4 Instrument

A Perkin Elmer Autosystem XL, equipped with a liquid autosampler and a flame ionization detector was used. The FAME samples were analyzed on a CP-Sil 88 capillary column (50 m, 0.32 mm ID 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer Chromeleon® version 7.

The analysis time was 60 min and the temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C for min, held at 160 °C for 25 min, ramped to 220 °C at 3 °C for min, held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 80 kPa.

8.4 Data analysis

The impact of the acclimation period on the fatty acid profile from liver of *T. bernacchii* was evaluated by comparing the variance components at 0 and 30 days. After 30 days of acclimation, the effect of time (0, 1, 5 and 10 days) and temperature (-1.8, 0.0, 1.0 and 2.0 °C) on the levels of fatty acids in liver of *T. bernacchii* was evaluated by using the experimental arrangement described in Table 8.1. The variables were modelled according to a quadratic polynomial of the form:

$$y = b_o + b_1 x_1 + b_2 x_2 + b_{12} x_1 \times x_2 + b_1^2 x_1^2 + b_2^2 x_2{}^2 \qquad \text{(Eq. 1)}$$

where y represent the concentration of fatty acid in mg g^{-1}, the terms x_1 and x_2 represent the variables time and temperature, respectively, the term b_o represents the intercept, b_1 and b_2 are the linear coefficients, b_{12} is the first order interaction effect coefficient and b_1^2 and b_2^2 are second order curvature effect coefficients. A detailed description of the levels of the variables time and temperature is given in Table 8.1. Before modeling the various fatty acids, their normality was evaluated by comparing the values of the experimental distribution with the corresponding points of the normal distribution using inverse normal plots.

A comprehensive explanation of the different steps involved in the development of the mathematical model for stearic acid (18:0) is given in Fig. 8.2. The various features of Fig. 8.2 are described by using roman numerals. The first step consisted of running in random order the experimental matrix (**i**), which contains duplicate conditions for time (x_1) and temperature (x_2), and determining the responses ($y_{18:0}$) which are expressed in mg g^{-1} of fatty acid (**ii**). Secondly, the modelling process is carried out by constructing a design matrix **(iii)** with a number of columns (6 columns) equivalent to the number of coefficients in Eq. 1 (6 coefficients). The six columns in (**iii**) are constructed as follows: the first column (x_0) is filled in with a numerical value

of 1 and it is used to estimate the intercept (b_0). The two subsequent columns (in grey), filled in with the experimental values of time (x_1) and temperature (x_2), are used to determine the coefficients b_1 and b_2. The fourth column (x_{12}) is the result of multiplying columns x_1 and x_2 and allows computing the coefficient b_{12}. The last two columns (x_1^2 and x_2^2) represent the square of the columns x_1 and x_2 and they are used to calculate the coefficients b_1^2 and b_2^2. The vector $y_{18:0}$ (**ii**) is regressed on the matrix (**iii**) and equation (**iv**) is computed. The validity of (**iv**) is confirmed by using the ratio between the lack of fit mean square and the pure error mean square at a 95% confidence level (Online resource 8.1) and described elsewhere (Araujo 2009). After validating model (**iv**) a matrix of estimated responses (**v**) is determined within the boundaries of the experimental ranges by substituting in (**iv**) the variables x_1 and x_2 (between 0 and 10 days and between -2 and 2°C respectively). Matrix (**v**) indicates for example that the concentration of 18:0 at 0-day/2°C (0.59 mg g^{-1}) is lower than 10-days/2°C (1.21 mg g^{-1}). To rapidly summarize the large amount of data in matrix (**v**) a graphical display of (**vi**) can be used. The graphical display in Fig. 8.2 is more practical and operable and shows the distribution of data through color change. Every color in (**vi**) represents areas with similar concentrations. For example, according to (**vi**) the brown and grey colors indicate concentration ranges of 0.5-1.0 mg g^{-1} and 0.5-1.0 mg g^{-1} respectively. In addition, a rapid inspection of (**vi**) allows concluding that condition 0-day/2°C exhibits a lower concentration than condition 10-days/2°C, without the need of a detailed examination of the numerical matrix (**iv**).

8.5 Results

The variance components for the fatty acid profiles of the caught fish in the Ross sea (control seawater) and the fish acclimatized for 30 days in a circulating seawater tank were determined and the experimental ratio between the lack of fit mean square and the pure error mean square (aka Fisher ratio or F-test) computed and compared against the tabulated F value of 18.51 (p = 0.05) for 1 and 2 degrees of freedom in the numerator and denominator, respectively. The results revealed the absence of statistical significant differences between the two types of controls (results not shown). The fatty acid profiles from liver of *T. bernacchii* at different times and temperatures conditions after 30 days acclimation are described in Table 8.1. The oleic acid

(18:1n-9) and docosahexaenoic acid (22:6n-3) exhibited the highest recorded concentrations, followed by palmitic acid and eicosapentaenoic acid. The inverse normal plots revealed a high degree of linearity for the various fatty acids (Online resource 8.2) confirming that the data in Table 8.1 were normally distributed. Quadratic polynomial models were generated by using Eq. 1 and the data in Table 8.1. The behavior of the saturated fatty acids (SFA) (14:0, 16:0, 18:0), monounsaturated fatty acids (MUFA) (16:1n-7, 18:1n-7, 16:1n-9, 18:1n-9, 20:1n-9, 22:1n-9) and polyunsaturated fatty acids (PUFA) (18:2n-6, 20:4n-6, 18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3) (mg g^{-1}) as a function of the variables time (days) and temperature (°C) is visualized in Fig. 8.3.

Myristic acid (14:0). At 0 and 1 days there was a continuous decrease in the concentration of 14:0 in the whole range of temperatures (-1.8, 0.0, 1.0 and 2.0°C). The behavior at 5 days between -1.8 and 0.0°C was characterized by a decrease in concentration around 35% with an additional reduction of approximately 10% when the temperature was increased from 0.0 and 1.0°C with not further change in concentration (0.77 ± 0.02 mg g^{-1}) between 1.0 and 2.0°C. A similar reduction in concentration (~40%) was observed at 10 days between -1.8 to 1.0°C.

Palmitic acid (16:0). There was a similar behavior to that observed for 14:0 in terms of reduction in concentration for the whole range of temperatures at 0 day (58%) and 1 day (49%) in the whole range of temperatures. The concentrations at 5 and 10 days remains almost constant (5.01 ± 0.64 and 4.42 ± 0.37 mg g^{-1}, respectively) in the whole range of temperatures.

Stearic acid (18:0). The behavior of stearic acid was similar to 16:0 and characterized by a continuous reduction in concentration when the temperature was raised from -1.8°C to 0.0°C (~25%), 1.0°C (~40%) and 2.0°C (~50%) at 0 and 1 day. At 5 and 10 days the concentration remains constant in the whole range of temperatures (1.16 ± 0.09 mg g^{-1}).

Palmitic oleic acid (16:1n-7). A dramatic decreasing in concentration was observed in the whole range of temperatures at 0, 1 and 5 days. The maximum reduction in concentration was 92% at 1 day and between -1.8 and 2.0°C. At 10 days the concentration remains almost constant in the whole range of temperatures (3.32 ± 0.39 mg g^{-1}).

Vaccenic acid (18:1n-7). At 0, 1 and 5 days there were significant reductions in the concentration of 18:1n-7 of approximately 70, 60 and 30%, respectively, while at 10

days the concentration remains almost constant (2.41 ± 0.15 mg g^{-1}) between -1.8 and 2.0°C.

Hypogeic acid (16:1n-9). The behavior of 16:1n-9 was similar to those observed for 14:0, 16:0 and 18:0 with a progressive reduction in concentration around 50, 60 and 70% at 0 day and 40, 50 and 55 % at 1 day when the temperature was increase 0.0, 1.0 and 2.0°C, respectively. Between 5 and 10 days the concentration remains almost constant (0.43 ± 0.05 mg g^{-1}) between -1.8 and 2.0°C.

Oleic acid (18:1n-9). This monounsaturated fatty showed a behavior similar to 14:0 and 18:1n-7 with significant reductions in its concentration in the ranges of 65-100, 50-80 and 30-40% at 0, 1 and 5 days, respectively in the whole range of temperatures. The concentration remains almost constant at 10 days (5.55 ± 0.53 mg g^{-1}) between -1.8 and 2.0°C.

Gondoic acid (20:1n-9). The behavior of 20:1n-9 resembles 14:0, 18:1n-7 and 18:1n-9. Significant reductions around 40-70, 35-60 and 20-30% at 0, 1 and 5 days, respectively and between -1.8 and 2.0°C were observed. There was not variation in concentration (0.69 ± 0.04 mg g^{-1}) at 10 days in this range of temperatures.

Erucic acid (22:1n-9). Erucic acid did not reveal significant changes in its concentration in the whole ranges of time and temperatures (0.21 ± 0.03 mg g^{-1})

Linoleic acid (18:2n-6). Linoleic acid revealed a similar reduction in concentrations at 0 and 1 day in the whole range of tested temperatures. The concentration remains constant (0.49 ± 0.06 mg g^{-1}) between 0 and 5 days at -1.8°C with a further decreased in concentration of around 30% at 10 days. It was also observed that at 5 and 10 days the concentration was independent of the temperature with average concentration values of 0.46 ± 0.07 and 0.32 ± 0.02 mg g^{-1}, respectively.

Arachidonic acid (20:4n-6). The model showed not significant changes in the concentration of arachidonic acid at different days and temperatures as reflected by the within day and within temperature averages. The former averages were 0.66 ± 0.05, 0.69 ± 0.05, 0.77 ± 0.05 and 0.73 ± 0.06 mg g^{-1} at 0, 1, 5 and 10 days, while the latter were 0.67 ± 0.03, 0.76 ± 0.04, 0.74 ± 0.06 and 0.67 ± 0.07 mg g^{-1} at -1.8, 0.0, 1.0 and 2.0°C, respectively.

α-Linolenic acid (18:3n-3). The concentration was independent of the temperature at 0 day (0.06 ± 0.01 mg g^{-1} between -1.8 and 0.0°C), 1 day (0.07 ± 0.01 mg g^{-1} between -1.8 and 1.0°C), 5 days (0.09 ± 0.01 mg g^{-1} between 0.0 and 2.0°C) and 10 days (0.06 ± 0.00 mg g^{-1} between -1.8 and 2.0°C). Out of the specified temperature ranges

for 0 and 1 day there was a decrease in concentration while for 5 days there was an increase in the concentration.

Eicosapentaenoic acid (20:5n-3). There was an almost constant increase in the concentration of around 30% between 0 and 10 days when the temperature was raised from -1.8 to 0.0°C. Further increases in temperature do not bring about changes in concentration. Between 0.0 and 2.0°C the average concentrations at 0, 1, 5 and 10 days were 3.14 ± 0.15, 3.58 ± 0.15, 4.60 ± 0.14 and 4.12 ± 0.13 mg g^{-1}, respectively.

Docosapentaenoic acid (22:5n-3). There was overall increase in concentration at the different temperatures and days. Increases in concentration of 18% (between 0 and 1 day), 54% (between 1 and 5 days) and 33% (between 5 and 10 days) were observed at -1.8°C. Identical increase patterns in concentration of 6% (0-1 day), 20% (1-5 days) and 15% (5-10 days) were observed at 0.0 and 1.0°C. At 2.0°C the increase pattern was 9% (0-1 day), 29% (1-5 days) and 19% (5-10 days).

Docosahexaenoic acid (22:6n-3). The response surface for 22:6n-6 displayed a behavior similar to 20:5n-3. There was average increase in concentration of 38% in the whole range of time (0-10 days) between -1.8 and 0.0°C. Between 0.0 and 2.0°C the average concentrations at 0, 1, 5 and 10 days were 4.56 ± 0.26, 5.41 ± 0.26, 7.32 ± 0.28 and 6.41 ± 0.30 mg g^{-1}, respectively.

8.6 Discussion

The ubiquitous species of stenothermal are characterized by their evolutionary adaptation to the extremely low temperatures of the Antarctic region, which is associated with a unique physiological adaptation but as well involves limited compensation capacities to the environmental changes. These species cope with the cold environment by several types of adaptations including, a reduction or loss of hemoglobin, higher mitochondrial densities, expression of anti-freeze glycoproteins, lack of heat shock response, variation in lipid class or changes in the FA composition (Eastman 1993; Pörtner and Playle 1998). The current research aims to answer a crucial question of ocean warming and its consequences on ecological interactions, by measuring changes in fatty acid profiles over time with variation in body temperature of Antarctic fish *T. bernacchii*.

There is a paucity in information regarding the behavior of the fatty acid profiles when the factors time and temperature are varied simultaneously. The majority of published studies are mainly concerned with the effect of the temperature on the concentrations of fatty acids without considering temporal changes (Ramesha and Thompson 1982; Dey et al. 1993; Buda et al. 1996; Lahdes et al. 2000; Brodte et al. 2008). The few reported studies on the simultaneous effect of time and temperature (Smith and Kemp 1971; Sellner and Hazel 1982; Hazel and Carpenter 1985; Copeman et al. 2013) have not considered the combined effect of these variables on the fatty acid concentrations, probably due to the very low number of degrees of freedom to judge for example the adequacy of potential mathematical models. The models for 14:0, 16:0 and 18:0 revealed that at 10 days and between 0.0 and 2.0°C there was a non-significant increase of approximately 4, 5 and 6% in absolute concentrations, respectively (Fig. 8.3). These increases in concentrations are equivalent to 14, 16 and 16% in relative units (computed from Online resource 8.3), respectively. These results are close to those reported by Brodte et al. (2008) who measured the fatty acid profiles in liver of Antarctic fish (*Pachycara brachycephalum*) and reported non-significant increase of 20, 16 and 25% for 14:0, 16:0 and 18:0 (measured in relative units), respectively at 120 days and between 0.0 and 2.0°C. Similarly, non-significant increments of 15 and 20% in relative concentration have been observed in temperate fish by Roche and Peres (1984) and Hazel (1979), respectively. In the current study, the content of SFA in the liver of the control group at -1.87°C was around 24.0% of total FA. The obtained value for hepatic SFA was comparable to those observed in *T. bernacchii* in other studies (Corsolini and Borghesi, 2017; Malekar et al. 2018). Upon higher temperatures, the relative concentration of SFA remained almost constant in the liver of *T. bernacchii,* over the studied acclimation period (1-10 days). Similarly, Brodte et al. (2008) found that the content of SFA in the liver of Antarctic fish did not change at higher temperatures (2, 4 and 6 °C) when compared to the control group (-0°C). Recently, Malekar et al. (2018) demonstrated also that the SFA composition of phospholipids in the liver of *T. bernacchii* remained constant at elevated temperatures during 14 days. Thus, the current and previous studies on the effect of temperature on SFA composition in *T. bernacchii* indicated that SFA might be less susceptible to changes under warmer conditions.

The observed decrease in concentration for 16:1n-7 between 0 and 5 days as the temperature increase has been also reported in different polar and temperate fish

species by evaluating the effect of increasing the temperature at specific times (Roche and Pérès 1984; Dey et al. 1993; Brodte et al. 2008). The reduction in concentration for 18:1n-7 and 18:1n-9 between 0 and 5 days in the investigated temperature range is in agreement with the results of Dey et al. (1993) and Roche and Pérès (1984) who reported a decreasing in concentration of 18:1n (not distinction was made between the two isomers) in temperate fish after comparing two experimental temperatures. Brodte et al. (2008) reported an astonishing increase of ~3000% for 18:1n-7 in the liver of Antarctic fish (difference between fish exposed to 0.0 and 4.0°C during 120 days) and a decrease of 39% in temperate fish (difference between fish exposed to 4.0°C and 6.0°C during 120 days). The behavior of 16:1n in livers of different temperate fish has been analyzed after one month acclimation period at two different temperatures (Roche and Pérès 1984; Dey et al. 1993). The results of these studies revealed not differences in concentration in seawater fish (Roche and Pérès 1984; Dey et al. 1993) but in freshwater fish (Dey et al. 1993). Similar results to those reported for seawater fish have been predicted by the model for 16:1n-9 (Fig. 8.3) where the concentration does not change significantly between 5 and 10 days in the range of -1.8 and 2.0°C. The predicted constant concentration at 10 days and between 0.0 and 2.0°C by the 20:1n-9 model (Fig. 8.3) for Antarctic fish *T. bernacchii* are in full agreement with studies on Antarctic fish *Pachycara brachycephalum* which observed not change in concentration after 120 days in the range of 0.0 and 2.0°C (Brodte et al. 2008).

When fish were immediately exposed to higher temperature (2.0°C after 1 day of exposure), the concentration of MUFA decreased in the liver of *T. bernacchii*, specifically the concentration of oleic acid (18:1n-9) decreased by 50 % compared to the control group (-1.8 and 2.0 °C after 1 day of exposure). Such an effect has been observed previously in many fish species including; *Pachycara brachycephalum*, *Zoarces viviparus* (Brodte et al. 2008) and *Salmo gairdneri* (Hazel 1979; Sellner and Hazel 1982). However, those results were observed at different temperature exposure time (from 3 days to 3 months) and in different organs fish species (liver, muscle, gill and kidney) (Smith and Kemp 1971; Sellner and Hazel 1982; Hazel and Carpenter 1985), whereas for *T. bernacchii*, the change in hepatic FA was observed immediately after 1 day of exposure to higher temperatures. Thus, the variation of MUFA composition in response to the temperature changes could occur differently depending on the tissue (Smith and Kemp 1971; Sellner and Hazel 1982; Hazel and Carpenter 1985), on the fish species physiology but also occurred at different time course.

The contour plots for the six PUFA predict non-significant changes in concentration at 1, 5 and 10 days between 0.0 and 2.0°C. The predictions are consistent with the report of Brodte et al. (2008) who determined these PUFAs in liver from Antarctic fish without any significant change in concentration in the same range of temperatures (0.0 and 2.0°C) after 120 days. Studies on temperate fish have also observed not change in the concentration of 18:2n-6 (Hazel 1979; Roche and Pérès 1984; Dey et al. 1993; Brodte et al. 2008), 20:4n-6 (Roche and Pérès 1984; Dey et al. 1993; Brodte et al. 2008), 18:3n-3 (Roche and Pérès 1984); 20:5n-3 (Brodte et al. 2008), 22:5n-3 (Hazel 1979) and 22:6n-3 (Hazel 1979; Dey et al. 1993; Brodte et al. 2008).

In their natural environment, marine organisms adapt their physiological function to cope with different stress factors. Change in temperature affects the function of lipids, where metabolic rate might be affected and perturb the fluidity of membrane through change in phospholipids but also the FA composition of tissues. There is an inverse correlation between ambient temperature and the content of unsaturated fatty acids (UFA) (Roche and Pérès 1984; Hazel 1990, 1995). The biochemical response of stenothermal species to lower temperature is an increase in the UFA in both the membrane and depot of lipids (Brodte et al. 2008; Malekar et al. 2018; Truzzi et al. 2018). The level of MUFA in liver of *T. bernacchii* under warmer conditions was reduced significantly, from 49% of total FA (at -1.87°C) to 42, 38 and 36% of total FA at 0.0, 1.0 and 2.0°C, respectively. This decrease in UFA composition was in line with the inverse relation between UFA and the temperature changes, as it has been demonstrated in different fish species (Hazel 1979; Sellner and Hazel 1982; Logue et al. 2000). On the other hand, this decrease in UFA composition might also serve as indicator for protection mechanisms against oxidative stress induced by higher temperature, as previous studies have shown (Crockett 2008; Vinagre et al. 2014; Machado et al. 2014). Conversely, the content of PUFA in the liver of *T. bernacchii* increased after the first day of exposure to higher temperatures, from 23% of total FA (-1.87°C) to 36, 43 and 40% of total FA at 0.0, 1.0 and 2.0°C, respectively. These results are somewhat surprising, since most of the studies reported a decrease in the PUFA composition at higher temperatures in the tissues of different fish species. (Hazel 1979, 1990; Tiku et al. 1996; Pernet et al. 2007). However, data provided by Brodte et al. (2008) demonstrated also an increase in PUFA levels by more than 70% in the liver of Antarctic fish upon higher temperature (from 0.0 to 4.0°C after 120 days

of exposure). In this study, the authors concluded that the polar fish has already optimised their metabolism by using preferentially MUFA over the PUFA, when the temperature change (Bordte et al., 2008). Moreover, in the present study, the effect of increasing the temperature on the liver FA profile was investigated after 1, 5 and 10 days, although most of the studies reported a decrease in the PUFA composition at higher temperatures after a long period of exposure (Smith and Kemp 1971; Sellner and Hazel 1982; Hazel and Carpenter 1985). Thus, the effect seen on the PUFA compositon in the current study might be not related to the temperature changes, and also a long period exposure to higher temperatures should be considered.

After 5 days of exposure to higher temperature (0.0, 1.0 and 2.0°C), the FA composition in the liver of *T. bernacchii* were similar to the control group. Indeed, FA composition in the liver of Antarctic fish contained the same concentration as in their natural environment (-1.8 °C). Therefore, these results confirmed previous findings, where both polar and temperate fish, had the ability to rapidly adapt to the new environmental conditions (Seebacher et al. 2005; Bilyk and DeVries 2011). Hence, *T. bernacchii* maintained the capacity for new environmental conditions by fluctuations on their FA metabolite rate. These Antarctic fish species has the abilities for rapid adaptation to changing conditions.

8.7 Conclusions

The fact that the computed models for Antarctic *T. bernacchii* predict changes that are in agreement with well-documented studies not only on polar but also on temperate fish might indicate that polar and temperate teleosts will respond in a similar way to global warming, which in turn might indicate the existence of a common underlying model for teleosts.

To the best of our knowledge, this is the first report modelling the changes in fatty acid concentration as a function of systematic and simultaneous variations in time and temperature.

8.8 Acknowledgements

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8.9 Figures, Tables and Online resources

Figure. 8.1 Location of the sampling point at the Mario Zuchelli Station (74°42'052" South, 164°02'267" East), Antarctic region

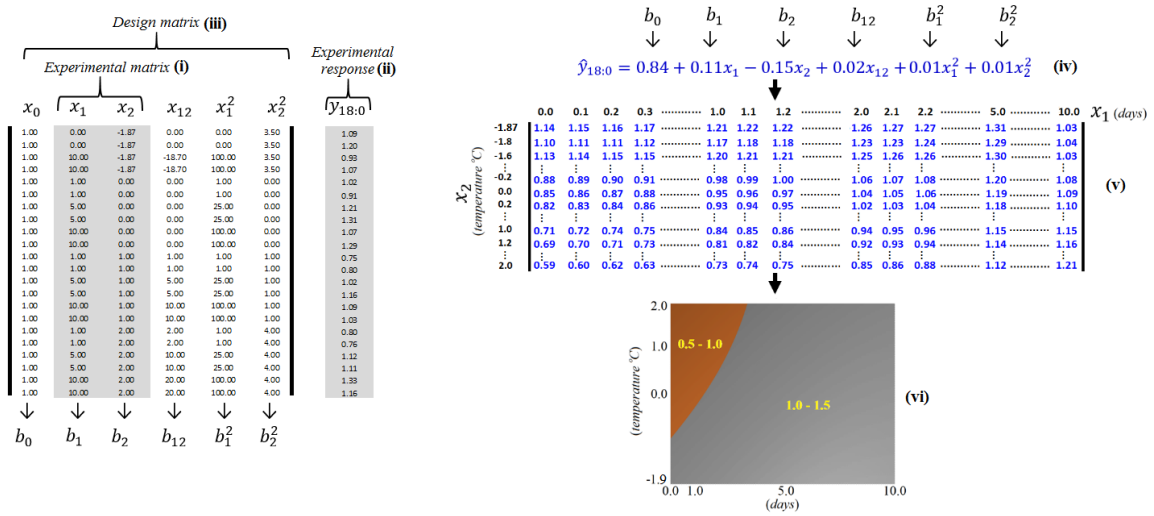


Figure. 8.2 Steps involved in the development of the mathematical model for stearic acid (18:0). The experimental matrix (i) is proposed and the corresponding responses (ii) are obtained. A design matrix (iii) is constructed and the equation (iv) is computed by regressing (ii) on (iii). The validity of (iv) is confirmed statistically (see Online resource 1) and a matrix of estimated responses (v) is generated within the experimental limits of the variables $\boldsymbol{x_1}$ (0.0-10.0 days) and $\boldsymbol{x_2}$ (-2.0-2.0°C). The large amount of data in (v) is plotted and summarize in (vi). The colors in (vi) represent areas with similar concentrations. For example, the brown and grey colors indicate concentration ranges of 0.5-1.0 mg g^{-1} and 0.5-1.0 mg g^{-1}, respectively. For a more detailed explanation see section *Data analysis*

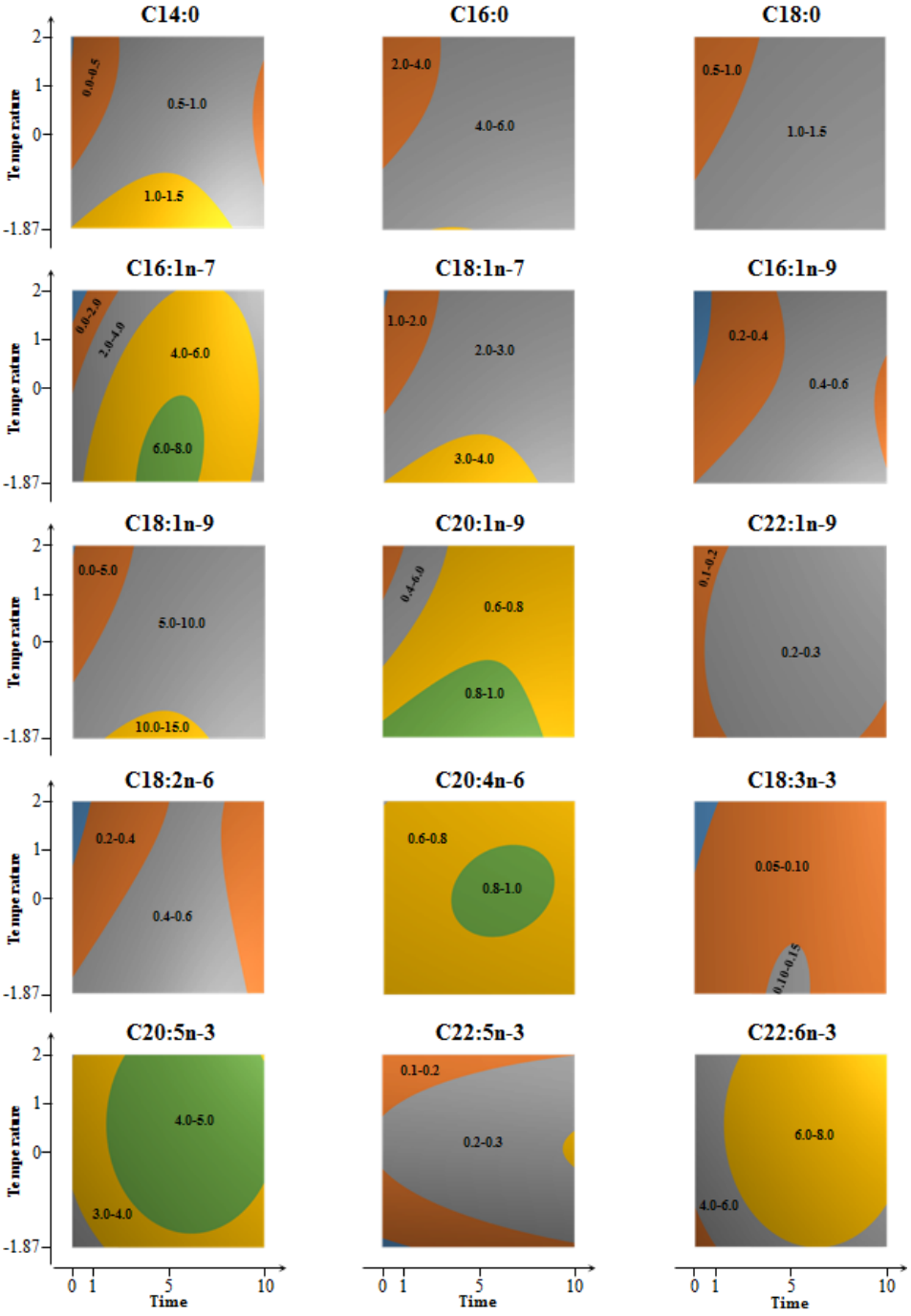


Figure. 8.3 Mathematical models describing the variation in fatty acid concentration (colored areas in mg g^{-1}) in liver of *T. bernacchii* as a function of time and temperature

Table 8.1 Absolute concentrations of fatty acids (mg g^{-1}) in the liver of *T. bernacchii*, sampled at day 0 (-1.87°C control) and at different times (1, 5 and 10 days) and temperatures (0, 1 and 2°C)

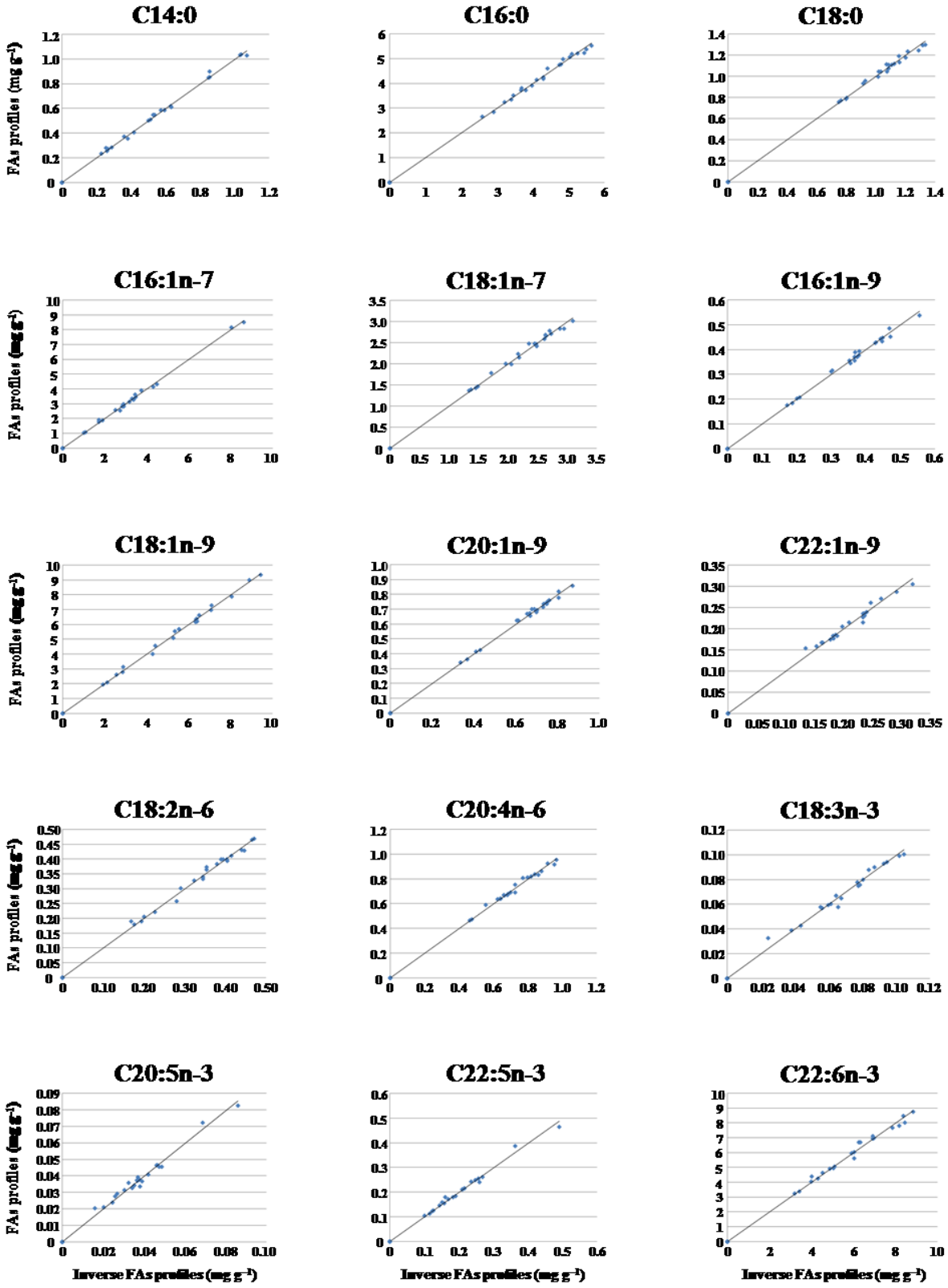
Time (days)	0	1	5	10	1	5	10	1	5	10
Temperature (°C)	-1.87	0.00	0.00	0.00	1.00	1.00	1.00	2.00	2.00	2.00
14:0	0.96±0.15	0.39±0.04	1.03±0.01	0.58±0.07	0.25±0.02	0.51±0.01	0.32±0.09	0.28±0.02	0.85±0.00	0.60±0.04
16:0	5.36±0.39	3.82±0.22	5.13±0.16	4.91±0.72	2.75±0.23	4.2±0.13	3.62±0.24	3.3±0.13	4.75±0.03	5.17±0.46
18:0	1.14±0.08	0.96±0.07	1.26±0.07	1.18±0.15	0.77±0.03	1.09±0.10	1.06±0.04	0.78±0.03	1.11±0.01	1.24±0.12
18:3n-3	0.07±0.01	0.07±0.01	0.08±0.00	0.09±0.02	0.06±0.00	0.10±0.01	0.05±0.03	0.04±0.00	0.09±0.01	0.06±0.00
20:5n-3	2.5±0.14	4.19±0.16	3.94±0.05	4.51±0.87	2.94±0.14	4.71±0.5	4.76±0.61	3.44±0.1	5.18±0.16	3.19±0.02
22:5n-3	0.11±0.01	0.25±0.02	0.21±0.00	0.43±0.09	0.12±0.00	0.25±0.01	0.21±0.07	0.15±0.01	0.21±0.00	0.18±0.01
22:6n-3	3.31±0.16	6.95±0.04	5.98±0.09	7.35±1.55	4.14±0.22	7.39±0.67	7.25±1.31	4.98±0.16	8.60±0.34	4.79±0.36
18:2n-6	0.41±0.04	0.38±0.04	0.40±0.01	0.40±0.07	0.21±0.02	0.39±0.02	0.22±0.08	0.18±0.01	0.47±0.00	0.33±0.01
20:4n-6	0.65±0.04	0.94±0.04	0.67±0.01	0.86±0.13	0.47±0.01	0.84±0.06	0.79±0.10	0.68±0.03	0.83±0.02	0.64±0.00
16:1n-9	0.36±0.02	0.33±0.04	0.37±0.01	0.42±0.07	0.21±0.00	0.43±0.00	0.34±0.05	0.18±0.01	0.44±0.00	0.51±0.06
18:1n-9	7.57±0.69	4.83±0.60	9.16±0.39	5.87±0.74	2.71±0.20	5.94±0.58	3.57±1.00	2.02±0.13	6.36±0.02	6.79±0.40
20:1n-9	0.84±0.05	0.69±0.05	0.74±0.01	0.74±0.09	0.35±0.02	0.68±0.02	0.65±0.07	0.42±0.01	0.76±0.00	0.72±0.04
22:1n-9	0.18±0.02	0.24±0.00	0.24±0.00	0.28±0.05	0.17±0.01	0.22±0.02	0.18±0.07	0.18±0.00	0.28±0.02	0.22±0.03
16:1n-7	3.05±0.19	2.70±0.29	8.32±0.42	3.88±0.61	1.81±0.12	3.39±0.13	2.23±0.71	1.04±0.05	2.85±0.04	4.11±0.52
18:1n-7	2.90±0.27	2.07±0.16	2.69±0.06	2.65±0.42	1.39±0.08	2.53±0.12	1.88±0.24	1.43±0.08	2.46±0.01	2.76±0.17

The values are expressed as average ± standard deviation (n=2)

Online resource 8.1 Statistical analysis used to judge the validity of the proposed models in Fig. 3. The example, corresponds to stearic acid (18:0) and the estimated response ($\hat{y}_{18:0}$) was determined by substituting x_1 and x_2 in $\hat{y}_{18:0} = 0.84 + 0.11x_1 + 0.15x_2 + 0.02x_{12} + 0.01x_1^2 + 0.01x_2^2$. The criteria for model acceptability was fulfilled when $F_{experimental} < F_{theoretical}$. The $F_{theoretical}$ corresponds to a tabulated value of 3.204 at the 95% confidence level and 5 and 11 degrees of freedom

Time	Temperature	Response (mg g^{-1})			Square errors		
(days)	(°C)	Experimental	Estimated	Average	Residual	Pure	Lack-of-fit
x_1	x_2	$y_{18:0}$	$\hat{y}_{18:0}$	$\bar{y}_{18:0}$	$(y_{18:0} - \hat{y}_{18:0})^2$	$(y_{18:0} - \bar{y}_{18:0})^2$	$(\bar{y}_{18:0} - \hat{y}_{18:0})^2$
0.00	-1.87	1.09	1.14	1.14	0.003	0.003	0.000
0.00	-1.87	1.20	1.14	1.14	0.003	0.003	0.000
10.00	-1.87	0.93	1.03	1.00	0.010	0.005	0.001
10.00	-1.87	1.07	1.03	1.00	0.002	0.005	0.001
1.00	0.00	1.02	0.95	0.96	0.004	0.003	0.000
1.00	0.00	0.91	0.95	0.96	0.002	0.003	0.000
5.00	0.00	1.21	1.19	1.26	0.000	0.003	0.005
5.00	0.00	1.31	1.19	1.26	0.015	0.003	0.005
10.00	0.00	1.07	1.09	1.18	0.001	0.012	0.007
10.00	0.00	1.29	1.09	1.18	0.037	0.012	0.007
1.00	1.00	0.75	0.84	0.77	0.007	0.001	0.004
1.00	1.00	0.80	0.84	0.77	0.001	0.001	0.004
5.00	1.00	1.02	1.15	1.09	0.018	0.005	0.004
5.00	1.00	1.16	1.15	1.09	0.000	0.005	0.004
10.00	1.00	1.09	1.15	1.06	0.004	0.001	0.008
10.00	1.00	1.03	1.15	1.06	0.013	0.001	0.008
1.00	2.00	0.80	0.73	0.78	0.005	0.000	0.003
1.00	2.00	0.76	0.73	0.78	0.001	0.000	0.003
5.00	2.00	1.12	1.12	1.11	0.000	0.000	0.000
5.00	2.00	1.11	1.12	1.11	0.000	0.000	0.000
10.00	2.00	1.33	1.21	1.24	0.015	0.008	0.001
10.00	2.00	1.16	1.21	1.24	0.003	0.008	0.001
			Σsum of errors →		0.145	0.080	0.065
			Degrees of freedom →		16	11	5
			Variance terms →		0.009	0.007	0.013
			$F_{experimental}$ →			1.774	

Online resource 8.2 Inverse normal plots for the various fatty acid profiles depicted in Table 1



Online resource 8.3 Relative concentrations of fatty acids (%) in the liver of *T.bernacchii,* sampled at day 0 (-1.87°C control) and at different time (1, 5 and 10 days) and temperature (0, 1 and 2°C)

Time (days)	0	1	5	10	1	5	10	1	5	10
Temperature (°C)	-1.87	0.00	0.00	0.00	1.00	1.00	1.00	2.00	2.00	2.00
14:0	3.17±0.26	1.31±0.06	2.50±0.08	1.65±0.06	1.30±0.10	1.52±0.00	1.14±0.12	1.42±0.02	2.35±0.03	1.88±0.04
16:0	17.7±0.08	12.9±0.04	12.4±0.05	14.0±0.26	14.6±0.89	12.6±0.16	13.2±1.4	16.9±0.04	13.2±0.15	16.1±0.72
18:0	3.77±0.02	3.25±0.05	3.05±0.09	3.35±0.12	4.10±0.09	3.25±0.24	3.87±0.54	3.99±0.02	3.09±0.02	3.88±0.22
18:3n-3	0.23±0.01	0.24±0.02	0.19±0.00	0.27±0.00	0.31±0.01	0.28±0.03	0.16±0.08	0.21±0.01	0.26±0.00	0.18±0.00
20:5n-3	8.27±0.18	14.1±0.33	9.52±0.12	12.7±0.36	15.6±1.1	14.1±1.2	17.3±0.83	17.6±0.22	14.4±0.19	9.96±0.52
22:5n-3	0.36±0.01	0.85±0.03	0.51±0.00	1.20±0.05	0.66±0.03	0.75±0.03	0.75±0.12	0.77±0.02	0.59±0.02	0.55±0.06
22:6n-3	10.9±0.33	23.5±1.6	14.5±0.59	20.8±0.95	22.0±1.6	22.1±1.6	26.2±0.12	25.4±0.25	23.8±0.50	15.0±1.8
18:2n-6	1.37±0.01	1.28±0.04	0.98±0.06	1.13±0.00	1.13±0.08	1.17±0.07	0.80±0.15	0.94±0.02	1.29±0.01	1.04±0.01
20:4n-6	2.15±0.03	3.17±0.07	1.63±0.08	2.45±0.04	2.49±0.00	2.50±0.13	2.88±0.16	3.47±0.01	2.29±0.00	2.00±0.10
16:1n-9	1.20±0.40	1.10±0.06	0.90±0.00	1.19±0.01	1.09±0.00	1.28±0.03	1.21±0.05	0.92±0.01	1.23±0.01	1.59±0.12
18:1n-9	25.0±0.35	16.3±1.0	22.2±0.36	16.7±0.67	14.4±0.75	17.8±2.1	12.8±1.4	10.3±0.24	17.6±0.28	21.2±0.31
20:1n-9	2.77±0.06	2.33±0.04	1.79±0.02	2.11±0.10	1.87±0.08	2.04±0.03	2.37±0.18	2.14±0.02	2.10±0.03	2.24±0.30
22:1n-9	0.58±0.02	0.80±0.04	0.57±0.00	0.80±0.01	0.91±0.07	0.66±0.04	0.65±0.14	0.94±0.02	0.77±0.04	0.68±0.11
16:1n-7	10.1±0.15	9.11±0.41	20.1±0.49	11.0±0.09	9.61±0.45	10.1±0.57	7.90±1.2	5.34±0.03	7.89±0.03	12.8±1.0
18:1n-7	9.56±0.17	7.00±0.12	6.49±0.02	7.52±0.07	7.40±0.26	7.57±0.48	6.83±0.33	7.30±0.11	6.83±0.09	8.61±0.15

The values are expressed as average ± standard deviation (n=2)

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9 Characterization of the fatty acids composition in cultivated Atlantic Bluefin Tuna (*Thunnus thynnus* L.) muscle by gas chromatography-mass spectrometry.

9.1 Abstract

An analytical method previously established in our laboratory, based on gas-chromatography-mass spectrometry (GC-MS), was applied for the first time to the determination of fatty acids (FAs) composition in the muscle of Atlantic Bluefin Tuna (ABFT) *Thunnus thynnus* L, caught in the Mediterranean Sea and farmed for several months, with the aim of demonstrating the feasibility of such measurements and to improve knowledge about the influence of size and sex on the FAs composition. Although no significant differences (in terms of quality of the product) were found in relation to sex, males and females showed a different correlation of FAs composition with weight. In male, a statistically significant linear correlation was found for 14:0, 16:0, 18:1n-9, 20:1n-9, 22:1n-9 (positive correlation), and for 20:4n-6, 20:5n-3, 22:6n-3 (negative correlation). In females, a statistically significant polynomial correlation for specific FAs was highlighted: specimens with medium size showed the lowest content of 20:1n-9 and 22:1n-9, and the highest content of 20:4n-6, 20:5n-3 and 22:6n-3. Therefore, males with size <150 kg and females with size from 150 to 250 kg appeared to be at greater nutritional value. This study demonstrated the practicality of the approach of the applied analytical methodology, and improved the knowledge of the influence of size on FAs composition of ABFT muscle, emphasizing a clear sex difference never found until now and representing a starting point to reach the best nutritional characteristics for both sexes in farmed tuna.

9.2 Introduction

Tuna is highly valued as a food fish around the world. Farmed tuna meat is higher in fat content than wild tuna (Topic Popovic et al. 2012), an excellent quality that makes it favored in the seafood market. Bred fishes are fed continuously and the excess of calories is accumulated in the form of triglycerides (TAG), rich in poly-unsaturated fatty acids (PUFAs), particularly in omega-3 fatty acids, such as eicosa-5,8,11,14,17-pentaenoic acid (EPA), and docosa-4,7,10,3,16,19-hexaenoic acid (DHA) (Topic Popovic et al. 2012; Wheeler and Morrissey 2003), very important from a nutritional point of view (Albert et al. 2002; Sidhu 2003; Simopoulos 2008; American Heart Association 2017). Moreover, tuna muscle shows a high ratio omega-3/omega-6 that is beneficial for health (Økland et al. 2007).

It is generally accepted that FA composition of lipids is species specific. However, the composition may vary greatly within a species. This variation is caused by both external factors, including habitat, temperature and salinity (Farkas et al. 1980; Saito et al. 1997), season capture (Saito et al. 1997; Jensen et al. 2007; Ould Ahmed Louly et al. 2011), diet (Saito et al. 1996; Öezogul and Öezogul 2007; Celik 2008; Morais et al. 2015), and internal factors such as physiological conditions (migration and reproduction) and age (Ishihara and Saito 1996; Kiessling et al. 2001; Nakamura et al. 2007; Mourente et al. 2015). In this regard, very few studies have been performed until now to relate lipid content of muscle of Atlantic Bluefin tuna (ABFT) to age and sex: total lipid content seems to be influenced by size (Ishihara and Saito 1996; Wheeler and Morrissey 2003), whereas DHA content does not seem to be affected by maturity (Ishihara and Saito, 1996). Other studies mainly regard variation of lipid content during oocytes maturation (Knapp et al. 2014), or during juvenile's growth and their calorie needs (Goñi and Arrizabalaga 2010; Logan et al. 2015), but until now no evidence on the lipid fatty acids composition of ABFT muscle in relation to size and sex are so far available.

To elucidate the influence of size and sex on FAs composition, it is necessary to keep constant other variables such as diet and environmental conditions. For this purpose, farmed tuna specimens, living in the same habitat at the same environmental conditions and diet for several months, satisfy all the required conditions and it is possible to relate FA composition to sex or size.

Generally, classical lipid extraction (Folch et al. 1957; Bligh and Dyer 1959) and gas chromatography coupled with flame ionization detector are used for the analysis of FAs in tuna meat (Mourente et al. 2002; Nakamura et al. 2007; Al-Busaidi et al. 2015, Hernández-Martínez et al. 2016), and data are reported as percentage of each FA *vs* total fatty acids. Recently, we optimized an analytical method for the determination of FAs composition in fish muscle, that involves a microwave-assisted extraction of lipids from a lyophilized sample, a derivatization of lipid extract to FAMEs, and their separation and identification by gas-chromatography-mass spectrometry (GC-MS) (Truzzi et al. 2017); this method allows identifying the absolute variation of every single fatty acid. We demonstrated that the microwave-assisted lipid extraction requires a minimum sample handling and a fast and reliable extraction at low temperatures that minimize fatty acids peroxidation, to which mono- and poly-unsaturated fatty acids are particularly sensitive. We applied this methodology for the first time to the analysis of FAs in the muscle of ABFT, *Thunnus thynnus* L., to demonstrate the feasibility and the practicality of this analytical methodology on the analysis of FAs composition in tuna muscle, and to improve knowledge on the influence of size and sex on FAs composition of muscle of farmed ABFT. Knowledge of lipid composition related with sex and size may be useful for the farmers to produce tuna with the best FA composition and excellent quality meat, satisfying the market preferences and increasing the value of the product.

9.3 Material and methods

9.3.1 Experimental design and animal sampling

ABFT *Thunnus thynnus* specimens were caught by purse seine from spawning grounds around the Mediterranean Sea during May-June 2015. Fishes were immediately moved into towing cages and transported over a period of several months to the tuna fish farm Fish and Fish Ltd (South–East of Malta). Fishes were fed with defrosted raw fish, as Pacific mackerel *Scomber japonicus*, Atlantic mackerel *Scomber scombrus*, and Atlantic herring *Clupea harengus*.

A total of forty ABFT specimens (20 male and 20 female) were obtained from the tuna fish farm in November 2015, during the post-spawning period. Curved fork length

(from the tip of the upper jaw to the fork of caudal fin) and total body weight were measured for all tuna fish sampled. The overall mean length was 228±33 cm, (males 240±31 cm, females 216±31 cm). The overall mean body weight was 238±93 kg, (males 279±86 kg, females 198±83 kg). Sex of fish was determined by examining gonads under a dissecting microscope. All fish were in the adult stage. Muscle samples were obtained from the upper part of the back, frozen immediately on dry ice, and then stored at -80°C until analysis. For each specimen, three independent samples of muscle (about 10 g each) were collected.

The animals were sampled under the guidelines Art 36, par.1 REg (EU) N°508/2014. The procedures did not include animal experimentation, so ethics approval is not necessary for accordance with the Italian legislation.

9.3.2 Fatty acids analysis

All solvents and reagents were of HPLC grade (see Truzzi et al. 2017). Each fish fillet was minced, homogenized (homogenizer MZ 4110, DCG Eltronic), and divided into aliquots of ~1 g each. Analyses were carried out on three aliquots *per* fish. Tissues were accurately weighed and freeze-dried (Edwards EF4, Crawley, Sussex, England) until constant weight. Lipid extraction was carried out on lyophilized powders following a Microwave-Assisted Extraction (MAE) according to a procedure of Truzzi et al. (2017).

Fatty acids were converted to their FAMEs and determined on an Agilent-6890 GC System coupled to an Agilent-5973N quadrupole mass selective detector, following the method optimized by Truzzi et al. (2017). The response factor for each FA was calculated against nonadecanoic acid methyl ester used as internal standard (19:0, 99.6%, Dr Ehrenstorfer Gmbh, Germany). Fatty acid quantification was carried out by calculating both the percentage of each FA *versus* total FAs and the mass fraction of fatty acids in g kg^{-1} of edible portion (fillet). For each sample, at least three runs were performed on the GC-MS.

The estimated limits of detection and limits of quantification, calculated as in Truzzi et al. 2014a, 2014b, ranged from ~4 µg mL^{-1} to ~22 µg mL^{-1}, and from ~13 µg mL^{-1} to ~66 µg mL^{-1}, respectively (Truzzi et al. 2014).

9.3.3 Nutritional indices

From the fatty acid profile (as percentage of each fatty acid *versus* total fatty acids), three nutritional indices were calculated, which attributed different weights to fatty acids depending on the different contribution of these to the promotion or prevention of cardiovascular disorders: atherogenicity index (AI) and thrombogenicity (TI) indicx (Ulbricht and Southgate 1991), and the Hypocholesterolemic to Hypercholesterolemic fatty acid ratios (HH) (Santos-Silva et al. 2002):

$$AI = [12:0 + (14:0 \times 4) + 16:0] / (\Sigma MUFAs + \Sigma PUFA\text{-}n\text{-}6 + \Sigma PUFA\text{-}n\text{-}3)$$

$$TI = \Sigma (14:0 + 16:0 + 18:0) / [(0.5 \times \Sigma MUFAs + 0.5 \times \Sigma(n\text{-}6) + 3 \times \Sigma(n\text{-}3) + (n\text{-}3/n\text{-}6)]$$

where: MUFAs are Mono-unsaturated Fatty Acids, PUFAs are Poly-unsaturated Fatty Acids, distinguished in PUFA-n-6 (sum of omega-6 PUFAs) and PUFA-n-3 (sum of omega-3 PUFAs).

$$HH = (18:1n\text{-}9 + 18:2n\text{-}6 + 20:4n\text{-}6 + 18:3n\text{-}3 + 20:5n\text{-}3 + 22:5n\text{-}3 + 22:6n\text{-}3) / (14:0 + 16:0)$$

Also, other nutritional indices such as n-3/n-6, PUFAs/SFAs, Unsaturated Fatty Acids/Saturated Fatty acids (UFA/SFAs), and EPA(20:5n-3)/DHA(22:6n-3) ratios, and the sum EPA+DHA, were calculated from the fatty acid profile.

9.3.4 Statistical analysis

Data were expressed as mean±standard deviation (SD) of 20 specimens *per* group. For each variable, the Student *t* test was applied to find significant differences between groups at the 95% confidence level. Relationships between variables were assessed using Pearson's correlation coefficient *r* (Steel et al. 1996). All statistical tests were performed with the statistical software STATGRAPHICS plus 5.1 (STATGRAPHICS Plus 2000).

9.4 Results and discussion

9.4.1 Lipid content

Tuna fishes showed a water content of 600±40 g kg^{-1}, with a mean of 610±20 g kg^{-1} for males and 600±60 g kg^{-1} for females (Fig. 9.1). Lipid content was 120±40 g kg^{-1}, with a mean of 110±20 g kg^{-1} for males, and 130±50 g kg^{-1} for females. Therefore, all the analyzed tuna specimens have a high-fat content, according to Olagunju et al. (2012). No significant differences were noted as regard water and lipid content in relation to size and sex. Our data were consistent with lipid percentage of Bluefin tuna captured in the mid-Adriatic Sea (Topic Popovic et al. 2012), and of Albacore tuna (*Thunnus alalunga*) found in the Pacific Ocean (Wheeler and Morrissey 2003).

9.4.2 Fatty acid composition

Fig. 9.2 shows an example of a chromatogram obtained from a muscle sample of ABFT: no overlappings between peaks were noted. Table 9.1 shows fatty acid composition, as percentage of total FAs and as g kg^{-1} fillet, of specimens grouped *per* sex. For greater clarity, only fatty acids with a content > 0.1 % *vs* total FAs were reported. No significant differences between males and females were found in the percentage of each FA *vs* total FAs. About SFAs, the main was 16:0 (palmitic acid, 14-15 %), as reported in literature (Öezogul et al. 2007; Ould Ahmed Louly et al. 2011; Topic Popovic et al. 2012), followed by 14:0 (myristic acid, ~6%) and 18:0 (stearic acid, 4-5%), both for males and female. Within MUFAs, the most abundant were 18:1n-9 (oleic acid, ~12%), 22:1n-9 (erucic acid, ~11%), 20:1n-9 (eicosenoic acid, ~8%), and 16:1n-7 (palmitoleic acid, ~7%). As well as palmitic acid, the higher amount of oleic acid found in analyzed farmed fishes was attributed to the feed mainly composed by herrings and mackerels (Jensen et al. 2007; Öezogul et al. 2007; Celik 2008). Moreover, all MUFAs cited above are biosynthesized from palmitic acid; consequently, a high palmitic acid percentage could determine a high content of its metabolites. The most abundant PUFAs were, in both male and female ABFT, 20:5n-3 (EPA, 13-14 %), and 22:6n-3 (DHA, 12-13 %): their sum (~26%) was higher than the sum found in

mid-Adriatic Bluefin tuna, i.e. ~19% and ~23% for farmed and wild tuna, respectively (Topic Popovic et al. 2012). The very low amount of arachidonic acid (20:4n-6) found in analyzed specimens (~1%), could be related to the low percentage of its precursor, the linoleic acid (18:2n-6). As a whole, fatty acids class that accounted for a most important percentage was, both for male and female, MUFAs (~42%), followed by PUFAs (30-31 %), and SFAs (~27 %). A high percentage of MUFAs is characteristic of fish from warm or temperate regions (De et al. 1993). Between PUFAs, the most represented were the omega-3 fatty acids (about 90% of total PUFAs), in agreement with literature data (Mourente et al. 2015).

In terms of absolute content (g kg^{-1} fillet) (Table 9.1), females showed a statistically significant higher content of 17:0, 20:0, 22:0, 17:1n-7, 20:2n-6 and 20:3n-6 than males (Table 9.1), but these changes did not produce significant differences in total SFAs, MUFAs and PUFAs since the above minor FAs had a content for each to 1%. Omega-3 showed an overall mean of ~27 g kg^{-1} fillet (no significant differences were noted between males and females), comparable with omega-3 content of Bluefin tuna from mid-Adriatic, 26 g kg^{-1} fillet (Topic Popovic et al. 2012), and of albacore tuna *Thunus alalunga* from West coast, 21-35 g kg^{-1} fillet (Wheeler and Morrissey 2003). In particular, the EPA+DHA sum ranged from ~21 g kg^{-1} fillet to ~32 g kg^{-1} fillet in males, and from ~17 g kg^{-1} fillet to ~32 g kg^{-1} fillet in females, with an overall mean of ~24 g kg^{-1} fillet and ~27 g kg^{-1} fillet, respectively (Table 9.1): no statistically significant differences were found between male and female.

Considering the overall data set, MUFAs, PUFAs, and SFAs contribute for ~41, ~30 and ~26 g kg^{-1} fillet, respectively. Our data are consistent with those found in ABFT from mid-Adriatic Sea (MUFAs 44.8 g kg^{-1} fillet, PUFAs 29.5 g kg^{-1} fillet), except for SFAs (34.9 g kg^{-1} fillet, Topic Popovic et al. 2012).

In addition to sex, FAs composition was also analyzed in relation to ABFT size. Individual correlation between FA and fish weight was studied. Results of correlation coefficients at 95% confidence intervals are shown in Fig. 9.3: only FAs that significantly correlated to fish weight were reported. For males, eight out of the total FAs with a content > 1% were found to have a statistically significant linear correlation with fish weight: 14:0, 16:0, 18:1n-9, 20:1n-9, and 22:1n-9 (positive correlation), and 20:4n-6, 22:6n-3, and 20:5n-3 (negative correlation). Overall, in males both total SFAs

and total MUFAs showed a statistically significant positive linear correlation with weight, going from ~24 % to ~32 % , and from ~38% to ~46% with increasing size, respectively; total PUFAs showed a statistically significant negative linear correlation with weight, going from ~38% to ~23% with size increase. We can conclude that, with weight increase, male specimens accumulate in their fat saturated fatty acids, reducing significantly the content of PUFAs, very important from a nutritional point of view. For females, only five out of the total FAs with a content > 1% were found to have a statistically significant correlation with fish weight. 20:1n-9 and 22:1n-9 (as well as total MUFAs) showed a polynomial positive correlation with weight, with the lowest percentage in female with medium size (150-250 kg); 20:4n-6, 20:5n-3 (EPA), 22:6n-3 (DHA) (as well as total PUFAs), showed a statistically significant negative polynomial correlation with weight. Therefore, females with medium size showed the highest percentage values of EPA and DHA. These results underlined that in females, weight seems to have a different influence on FAs composition respect to male specimens, showing a clear sex differences in FAs composition related with size.

9.4.3 Nutritional characteristics

Based on the chemical differences of FAs composition with specimen size, also the nutritional indices were analyzed in relation to weight. Results are shown in Table 9.2 and in Fig. 9.4, where only indices that significantly correlated to fish weight were reported.

Generally, as seen for FAs, males and females showed a different correlation of nutritional indices with weight: a significant linear correlation for males, and a significant polynomial correlation for females, In analyzed tuna specimens, a very high n-3/n-6 ratio was found (~9) and no significant differences were noted in relation to sex, but the highest n-3/n-6 ratio (~10, Fig. 9.4) was found in male with size < 150 kg and in females with medium size (from 150 kg to 250 kg). Nutritionists fixed a desirable ratio n-3/n-6 ≥5 in fish to improve nutritional value and to prevent diseases (Moreira et al. 2001); the high value of n-3/n-6 ratio found in farmed tuna bred in the Mediterranean Sea suggests a good meat quality. The sum percentage of EPA+DHA ranged from 20 % to 33 %, both in males and females, and the highest values were found in males with size < 150 kg and in females with medium size (from 150 kg to 250 kg). Generally,

wild Bluefin tuna fish is characterized by and higher DHA/EPA ratios (≥2), characteristic of a carnivore diet (Mourente et al. 2015; Topic Popovic et al. 2012). In our farmed tuna, this ratio was about 1, both for males and females (Table 9.2). This composition is consistent with data obtained by Topic Popovic et al. (2012), where DHA/EPA ratio was 1.2 in farmed tuna. Moreover, it can be noted that wild Bluefin tuna use preferentially, as source of metabolic energy, MUFAs, such as 18:1n-9, 20:1n-9, and even EPA, but not DHA, and this is probably one of the causes of a high DHA/EPA ratio (i.e. values of 2-3) (Mourente et al. 2015). In farmed tuna, probably the chronic motion insufficiency determines lower energy requirements, which reduces the metabolism of fatty acids, improving the meat characteristics of cultured fish, such as the major percentage of the essential omega-3 EPA.

The PUFAs/SFAs ratio (~1.1 for the whole data set) was similar to that found in ABFT from Gulf of Mexico (Hernández-Martínez et al. 2016). All specimens showed a ratio > 0.45, i.e the minimum recommended value to avoid the potential to raise blood cholesterol level (Departement of Health and Social Security 1984).

Concerning AI, TI, and HH indices, the results of the present study agreed with literature data (Al-Busaidi et al. 2015; Hernández-Martínez et al. 2016). Low values of AI (≤ 0.51) and TI (≤ 0.30) are beneficial to health (Ulbricht and Southgate 1991). TI values of ABFT analyzed were below the limit for all specimens (range ~0.16 to ~0.34, Table 9.2), whereas AI values (range ~0.45 to ~0.70) were below the limit only for males with a smaller size (<150 kg) and females with medium size (150-250 kg). The HH index was high, from ~1.5 to ~2.8, both for males and females (Table 9.2), indicating that a regular intake of this fish could produce hypocholesterolemic effects (Santos-Silva et al. 2002).

9.5 Conclusions

An analytical method based on gas-chromatography-mass spectrometry was applied for the determination of fatty acids in the muscle of Atlantic Bluefin Tuna, to improve knowledge on the influence of size and sex on FAs composition. This study demonstrated the feasibility and the practicality of the approach of the analytical methodology applied and improved the knowledge about the influence of specimen

size on FAs composition of muscle of *Thunnus thynnus* L. In particular, we found that the muscle of ABFT farmed in the Mediterranean Sea did not show substantial differences in lipid profile in relation to sex but, surprisingly, males and females showed a different correlation of FAs composition with size, highlighting a clear sex difference never found until now. Therefore, although in general ABFT has a very high value from a nutritional point of view, male with size <150 kg and female with size from 150 to 250 kg appear to be at greater nutritional value. These results represent a starting point to reach the best nutritional characteristics for both sexes in farmed tuna and could support the farmers to produce tuna with the best FA composition and good quality meat, increasing the value of this product.

9.6 Acknowledgements

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9.7 Figures, Tables and Supplementary materials

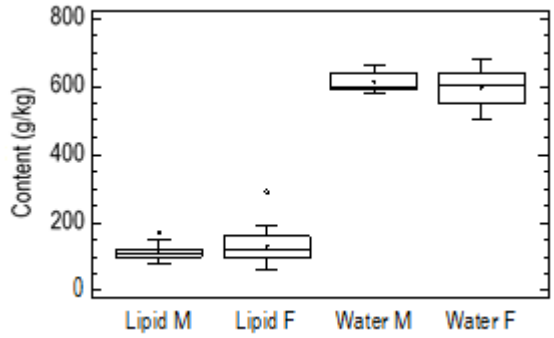

Figure 9.1. Box-and-whisker plot of lipid and water content in male (M) and female (F) of Atlantic Bluefin Tuna.

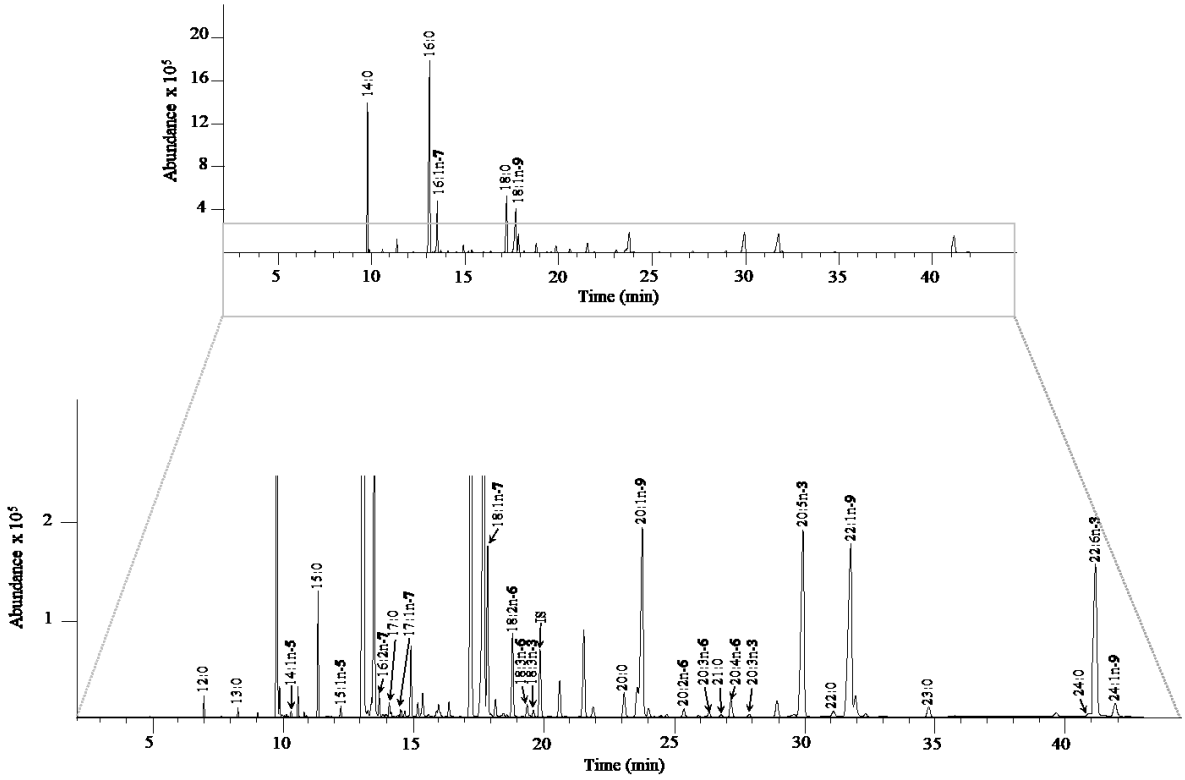

Figure 9.2. Example of a chromatogram of FAs composition from a muscle sample of Atlantic Bluefin Tuna.

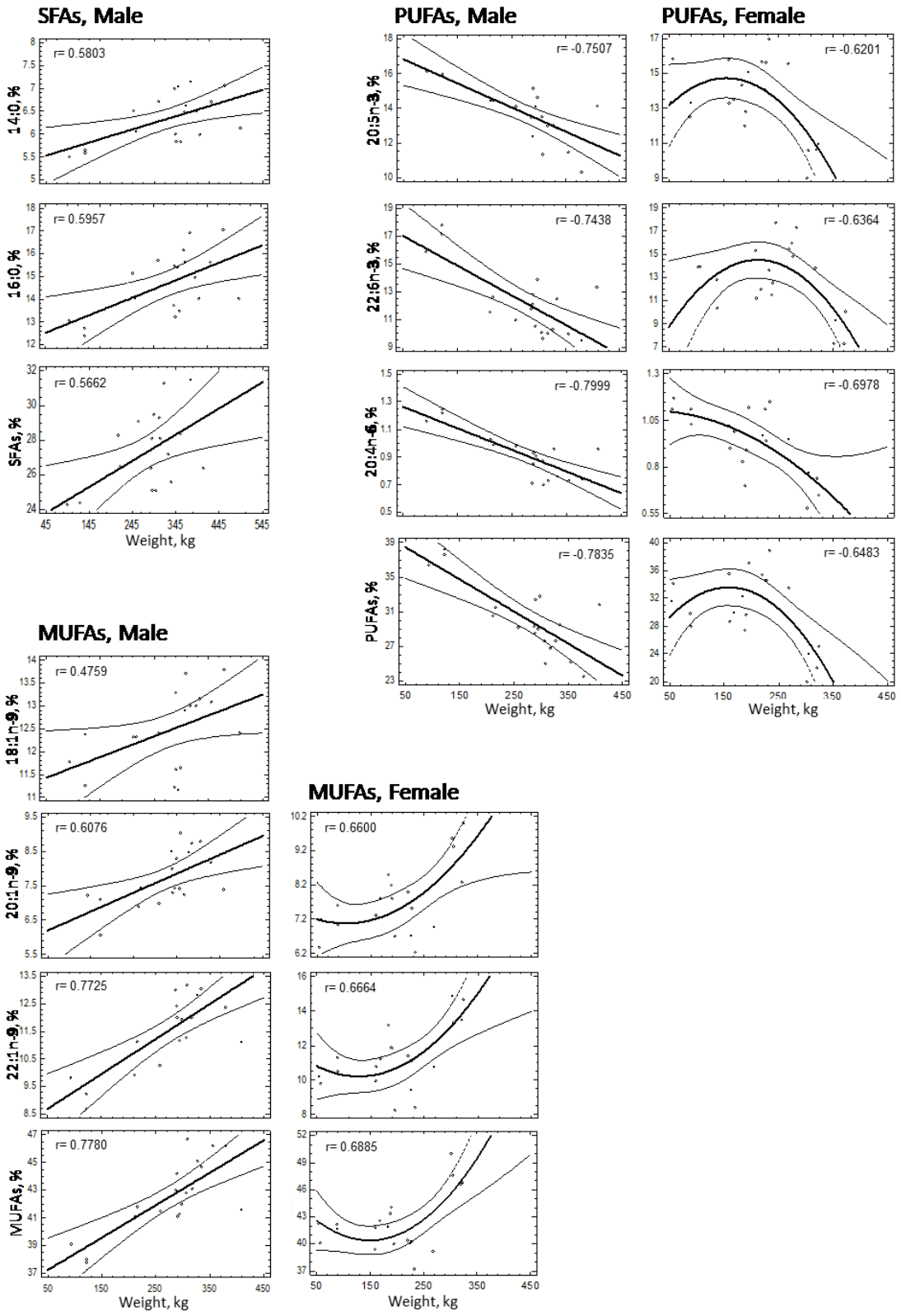

Figure 9.3. Correlation at 95% confidence interval between fatty acids content (% *vs* total FAs) and tuna body weight of male and female of Atlantic Bluefin Tuna. All correlation coefficients r indicate correspondence to significant correlation between fatty acids percentage *vs* total FAs and tuna weight ($P < 0.05$).

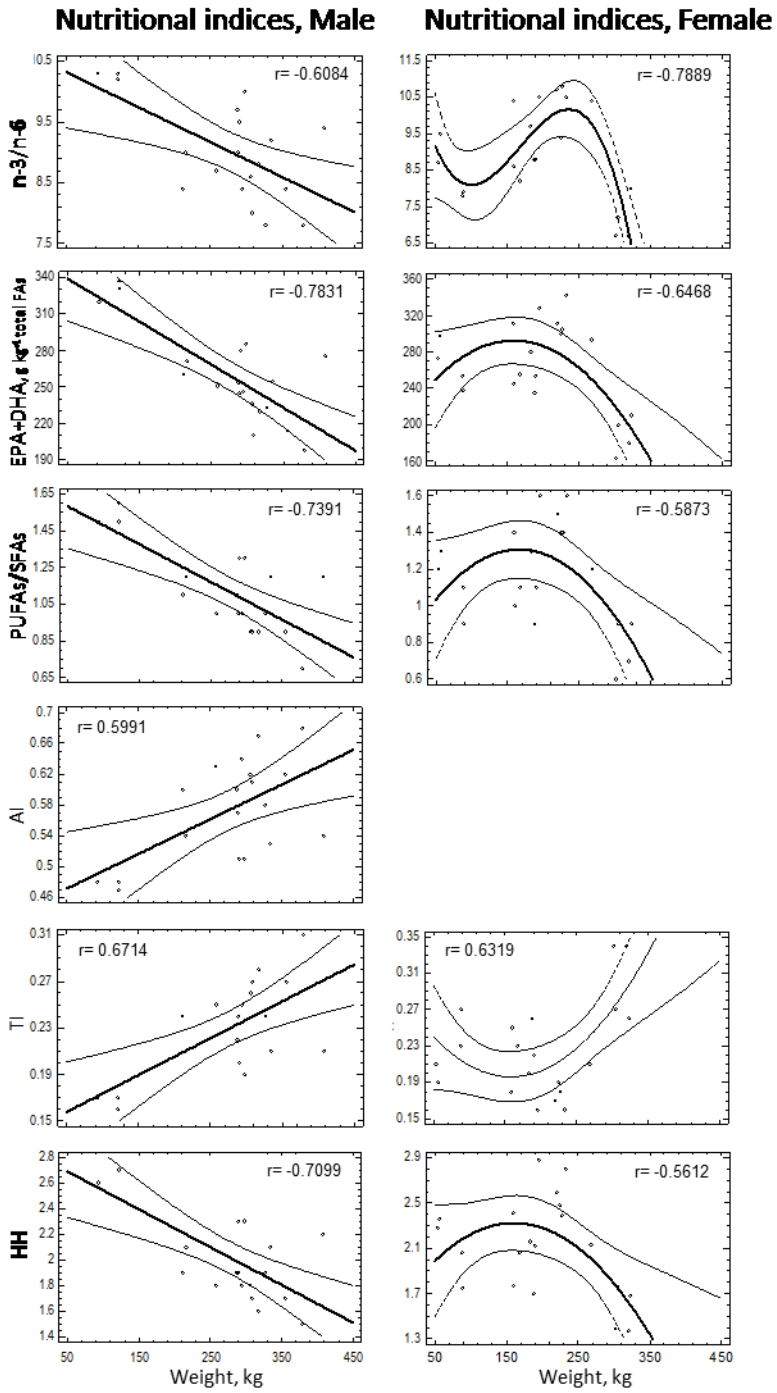

Fig. 9.4 Correlation of nutritional markers with tuna body weight of male and female of Atlantic Bluefin Tuna at 95% confidence interval. All correlation coefficients r indicate correspondence to significant correlation between nutritional markers and tuna weight ($P < 0.05$).

Table 9.1. Fatty acid (FA) composition (as % *vs* total FAs and as g kg^{-1} fillet) of the muscle of Atlantic Bluefin Tuna specimens separated for sex. Means within rows (of the same unit of measure) bearing different letters are significantly different (P<0.05).

Fatty Acids	male (n=20)		female (n=20)	
	% *vs* total FAs	g kg^{-1} fillet	% *vs* total FAs	g kg^{-1} fillet
14:0	6.3^{a}±0.5	5.7^{a}±0.7	6.1^{a}±0.7	6.2^{a}±1.5
15:0	0.53^{a}±0.04	0.48^{a}±0.05	0.53^{a}±0.05	0.54^{a}±0.13
16:0	15^{a}±1	13^{a}±2	14^{a}±1	15^{a}±4
17:0	0.45^{a}±0.04	0.40^{a}±0.04	0.47^{a}±0.07	0.47^{b}±0.10
18:0	4.5^{a}±0.4	4.1^{a}±0.4	4.7^{a}±0.5	4.8^{a}±1.1
20:0	0.35^{a}±0.03	0.31^{a}±0.04	0.36^{a}±0.06	0.37^{b}±0.09
22:0	0.17^{a}±0.02	0.15^{a}±0.02	0.18^{a}±0.03	0.18^{b}±0.04
Total SFAs^{+}	28^{a}±1	25^{a}±2	27^{a}±1	27^{a}±4
16:1n-7	6.7^{a}±0.4	6.0^{a}±0.7	6.6^{a}±0.6	6.7^{a}±1.7
17:1n-7	0.44^{a}±0.02	0.39^{a}±0.04	0.45^{a}±0.04	0.46^{b}±0.12
18:1n-9	12^{a}±0.8	11^{a}±1	13^{a}±1	13^{a}±4
18:1n-7	2.4^{a}±0.2	2.2^{a}±0.3	2.5^{a}±0.2	2.5^{a}±0.6
20:1n-9	7.7^{a}±0.8	7.0^{a}±1.2	7.7^{a}±1.0	7.9^{a}±2.4
22:1n-9	12^{a}±1	10^{a}±2	11^{a}±2	12^{a}±3
24:1n-9	1.1^{a}±0.1	1.0^{a}±0.2	1.2^{a}±0.2	1.2^{a}±0.3
Total MUFAs*	42^{a}±2	38^{a}±3	43^{a}±3	43^{a}±6
18:3n-3	1.0^{a}±0.1	0.92^{a}±0.16	1.0^{a}±0.1	1.1^{a}±0.33
20:3n-3	0.13^{a}±0.01	0.12^{a}±0.02	0.14^{a}±0.01	0.14^{a}±0.04
20:5n-3	14^{a}±2	12^{a}±2	13^{a}±2	14^{a}±5
22:6n-3	12^{a}±2	11^{a}±3	13^{a}±3	13^{a}±5
Total n-3 PUFAs	27^{a}±3	25^{a}±4	26^{a}±4	29^{a}±7
18:2n-6	1.5^{a}±0.1	1.3^{a}±0.2	1.5^{a}±0.1	1.5^{a}±0.4
18:3n-6	0.20^{a}±0.02	0.18^{a}±0.03	0.21^{a}±0.02	0.21^{a}±0.06
20:2n-6	0.24^{a}±0.01	0.22^{a}±0.03	0.25^{a}±0.02	0.25^{b}±0.08
20:3n-6	0.16^{a}±0.02	0.15^{a}±0.03	0.17^{a}±0.02	0.17^{b}±0.05
20:4n-6	0.91^{a}±0.2	0.83^{a}±0.21	0.94^{a}±0.2	0.96^{a}±0.32
Total n-6 PUFAs	3.0^{a}±0.2	2.7^{a}±0.3	3.1^{a}±0.2	3.1^{a}±0.5
16:2n-7	0.18^{a}±0.02	0.16^{a}±0.02	0.18^{a}±0.02	0.18^{a}±0.04
Total PUFAs	30^{a}±3	28^{a}±4	30^{a}±4	32^{a}±7
EPA+DHA	26^{a}±3	24^{a}±4	26^{a}±4	27^{a}±7

$^{+}$includes 12:0, 13:0, 21:0, 23:0, 24:0

*includes 14:1n-5, 15:1n-5

Table 9.2. Nutritional indexes (NI) of lipid fraction in Atlantic Bluefin Tuna muscle.

NI	Male	Female
n-3/n-6	9.1±1.1 (7.8-10.3)	8.9±1.3 (6.7-10.8)
DHA/EPA	0.86±0.2 (0.74-1.1)	1.0±0.3 (0.68-1.2)
PUFAs/SFAs	1.1±0.2 (0.75-1.62)	1.1±0.2 (0.64-1.64)
UFAs/SFAs	2.6±0.1 (2.2-3.2)	2.7±0.2 (2.1-3.3)
Atherogenicity Index	0.57±0.04 (0.47-0.68)	0.55±0.05 (0.44-0.72)
Thrombogenicity Index	0.23±0.02 (0.16-0.31)	0.22±0.02 (0.16-0.34)
Hypo-/Hypercholesterolemic Fatty Acid ratio	1.9±0.2 (1.5-2.7)	2.1±0.2 (1.4-2.9)

9.8 References

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10 A multidisciplinary approach to study the reproductive biology of wild prawns

10.1 Abstract

This work aims to provide deeper knowledge on reproductive biology of *Penaeus kerathurus* in a multidisciplinary way. Upon 789 examined females, 285 were found inseminated. The logistic equation enabled to estimate the size at first maturity at 30.7 mm CL for female. The Gono-Somatic Index (GSI) showed a pronounced seasonality, ranged from 0.80 ± 0.34 to 11.24 ± 5.72. Histological analysis highlighted five stages of ovarian development. Gonadal fatty acids analysis performed with gas chromatograph evidenced a pronounced seasonal variation; total lipids varied from 1.7% dry weight (dw) in Winter, to 7.2% dw in Summer. For the first time, a chemometric approach (Principal Component Analysis) was applied to relate GSI with total lipid content and fatty acid composition of gonads. The first two components (PC1 and PC2) showed that seasonality explained about 84% of the variability of all data set. In particular, in the period February-May, lipids were characterized by high PUFAs content, that were probably utilized during embryogenesis as energy source and as constituent of the cell membranes. During the summer season, gonads accumulated saturated FAs, that will be used during embryogenesis and early larval stages, while in the cold season total lipids decreased drastically and the gonad reached a quiescent state.

10.2 My contribution

10.2.1 Introduction

Information on the quantitative changes that occur in the different lipid classes and their fatty acids during the maturation period are scarce. Teshima and Kanazawa (1983) indicated that large quantities of lipids were necessary for the development of

Penaeus japonicus ovaries, and the amounts of ovarian lipids increased remarkably with increasing gonado-somatic index (GSI) values. These authors highlighted a quantitative ovarian lipid increasing during the sexual maturation, conversely their qualities did not vary so remarkably in the same prawn species.

10.2.2 Results lipid profile

From February 2015 to January 2016 (except for April when samples were not collected due to their seasonal migratory behavior) total lipids of gonad samples varied from minimum mean values of 1.8–1.7% dry weight (dw) in autumn and winter, to maximum mean values of 7.0% and 7.2% dw in spring and summer, respectively (Figure 10.1). Whereas total lipid content remained more or less constant in the period February-September, a significant drastic decrease was recorded in the period October-January, with a reduction of about 75%.

Supplementary Table S10.1 shows the complete lipid profile of gonad samples of prawns collected in different months. Annual fluctuations were found for almost all FAs, with statistically significant differences between months. Minor FAs, with a percentage <1%, were excluded from the statistical analysis, because they were present in a not significant quantity. Concerning the most important SFAs, the highest values were recorded in summer for myristic (14:0) and palmitic (16:0) acids, and in autumn/winter for margaric (17:0) and stearic (18:0) acids. Total SFAs ranged between 21.6% in May to 34.8% in July, with the highest values in summer, and decreasing values from October until May. Between MUFAs, the most represented show the highest values in summer (such as palmitoleic acid, 16:1n-7 and oleic acid, 18:1n-9), and in late winter/spring (*cis*-vaccenic acid, 18:1n-7). Total MUFAs ranged between 22.2% in February to 31.8% in September, with the highest values in summer. The essential fatty acid (EFA) 20:5n-3 shows the highest values in spring, and the lowest ones in Summer, whereas the other EFA 22:6n-3 shows the highest values in June/July, and the lowest ones from November to December. Overall, total n-3 PUFAs ranged from 27% in September to 39.7% in May, with the highest values from February to June, and the lowest from July to September. n-6 PUFAs ranged from 5.8% in August to 16.6% in February, with the highest values in February/March and very low values from June to September.

Statistical analysis revealed significant correlations between GSI and some SFA, MUFAs, n-6 PUFAs and n-3/n-6 ratio (Supplementary Table S10.1). In more detail, myristic acid (14:0), palmitic acid (16:0), total SFAs, palmitoleic acid (16:1n-7), oleic acid (18:1n-9), total MUFAs, and n-3/n-6 ratio, showed a significant positive correlation with GSI, while margaric acid (17:0), heptadecanoic acid (17:1n-7), eicosapentaenoic acid (20:5n-3), linoleic acid (18:2n-6), arachidonic acid (20:4n-6), n-6 PUFAs and total PUFAs showed a significant negative correlation with GSI (Supplementary Table S10.1).

To better understand the relationships between GSI, percentage of %TL and FAs composition, a multivariate analysis (PCA) was performed to reduce the dimensionality of the data set in few components, that summarize the information contained in the overall data-set. GSI and the %TL (*vs* dw) in gonads were included in the data-set, together with FAs with a percentage >1%.

Applying PCA to the data set (11 observations, 23 variables), it is possible to extract three significant, cross-validated principal components (Table 10.1). Together, they account for 90.8% of the variability in the original data. Table 10.1 shows the variance explained and the component loading matrix for the first three principal components extracted (PC1, PC2 and PC3). The higher the absolute value of a standardised coefficient, the more significant was the related selected variable. The first component is associated to GSI (positive coefficient), positively correlated with 14:0, 16:0, 16:1n-7, 18:1n-9, and total SFAs and MUFAs, as well as n-3/n-6 ratio, and negatively correlated with n-6 PUFA (such as 20:4n-6 and 18:2n-6), and the n-3 PUFA 20:5n-3, as well as total PUFAs (negative coefficients). The second component is dominated by the percentage of Total Lipids, positively correlated with the 22:6n-3, 18:1n-7 and n-3/n-6 ratio (negative coefficients), and negatively correlated with saturated fatty acids such as 15:0, 17:0, 18:0, and the monounsaturated 17:1n-7. GSI dominated the third component, that highlights the relationships between this index and some FAs, such as 15:0, 16:1n-7, 20:2n-6 (negative coefficients), and 18:0, 20:1n-9, 22:6n-3 (positive coefficients). This component explains only 7.21% of the variance, so we not considered this component in the discussion.

The Analysis of Similarity (ANOSIM) for test the differences between the four seasonal FAs data highlighted statistical significant differences ($p = 0.0074$) with

separation between level (R = 0.5689). The pairwise test indicated high separation level between summer *vs* winter and summer *vs* autumn (for all of them R = 1).

The Similarity Percentage Analysis (SIMPER) for all the seasonal pooled data highlighted a set of nine FAs that explain more than 90% of the cumulative dissimilarity between factors; in particular, three PUFAs (20:4n-6, 18.36%; 20:5n-3, 17.22%; 22:6n-3, 10.67%) explain 46.25% of the cumulative dissimilarity, followed by three SFAs (16:0, 14:0, 18:0) explaining 24.57% and three MUFAs (16:1n-7, 18:1n-9, 18:1n-7) explaining the remaining 19.81% of the dissimilarity between factors. The seasonal pairwise SIMPER showed a contribution of two PUFAs as the major suppliers of dissimilarity in all comparison, ranged from 29.83% between winter *vs* autumn (20:5n-3, 20:4n-6) and 42.72% in spring *vs* autumn (22:6n-3, 20:4n-6).

10.2.3 Discussion

The size increase of the oocytes was correlated with an increase in total lipids as already reported for many penaeid species, including *P. kerathurus* (Mourente and Rodríguez 1991; Teshima and Kanazawa 1983). Total lipids increased from 1.7% in winter to 7.0% in spring and to 7.2% in summer. Consequently, the GSI ranged from 0.8 (December) to 11.2 (July), confirming July as the reproductive peak for this species (summer, from May to September).

From the biplot reported in Figure 10.2, showing *loadings* and *scores* plots simultaneously, a relevant effect of seasonality was highlighted for this species. In particular, PC1 and PC2 showed that seasonality explained about 84% of the variability of all data-set: an annual cycle from February 2015 to January 2016 can be noted. The percentage of total lipids was high from February to March, and remained substantially constant, apart from a peak in June, but FAs composition changed in relation to the different months considered, because of the ovarian development. In particular, in the period February-May, lipids were characterized by a high PUFAs content, particularly FAs of the n-3 series. The Analysis of Similarity highlighted statistical significant differences with separation between level, indicating differences between the seasonal FAs composition, particularly evident between summer *vs* winter and summer *vs* autumn.

The statistical analysis of Similarity Percentage revealed in general a pool of FAs that have been defined the highest dissimilarity between factors. In more detail, in each seasonal pairwise comparison, two PUFAs were identified as the most influential source of dissimilarity among the seasons.

For *P. japonicus*, Teshima and Kanazawa (1982) highlighted a high lipid content in the ovaries, these molecules decreased to low level at nauplius and zoea stages, then remained roughly constant during the subsequent stages to post-larval. The same study verified that phospholipids (PL) and triglycerids (TG) were the major lipid classes in the ovaries and larvae, suggesting an important role of that these lipid classes as energy source and as constituent of the cell membranes in the ovaries and during embryogenesis. In the present study, a clear differentiation in a pre-spawning and a post-spawning phase was detected. During the summer season, the mature oocytes accumulated FAs that were used during embryogenesis and early larval stages, while in the cold season total lipids decreased drastically and the gonad reached a quiescent state.

In this species approximately 65% of fatty acids of the total ovarian lipids were conveyed to eggs during spawning; the fatty acids stored as triacylglycerol (TAG) in the midgut gland could play an important reserve role in the case of prolonged starvation before or during maturation or moulting (Dall 1981). Middleditch et al. (1980) stated that the increase in PUFAs concentration in the TAG fraction of both ovary and midgut gland could reinforce the theory that long-chain fatty acids are necessary for vitellogenesis of penaeids.

The present work introduces new aspects related to the species, such as a pronounced seasonality of FAs composition strongly related to their gonadal maturation. The reproductive event needs an extraordinary amount of energy, prerequisite as an investment in the progeny.

10.3 Conclusions

A multidisciplinary approach was used in the present study to have a better knowledge about *P. kerathurus* repro- ductive cycle. For the first time, a chemometric approach was applied to GSI data, total lipids and fatty acid composition of gonads of *P*. *kerathurus*, highlighting a relevant effect of seasonality for this species. These results, together with classical microscopical analysis and fisheries data, allowed to obtain a better characterization of the annual reproductive cycle of *P*. *kerathurus*.

10.4 Figures, Tables and Supplementary materials

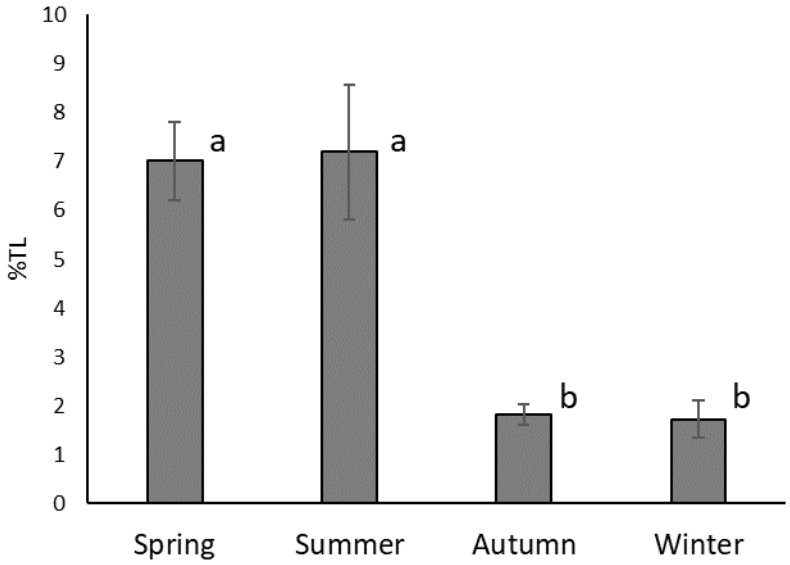

Figure 10.1. Total lipids concentration (%±SD, Standard Deviation) in *P. kerathurus* female gonads samples, in different seasons, from February 2015 to January 2016 (April 2015 excluded). Superscript letters indicate significant differences ($p < 0.05$).

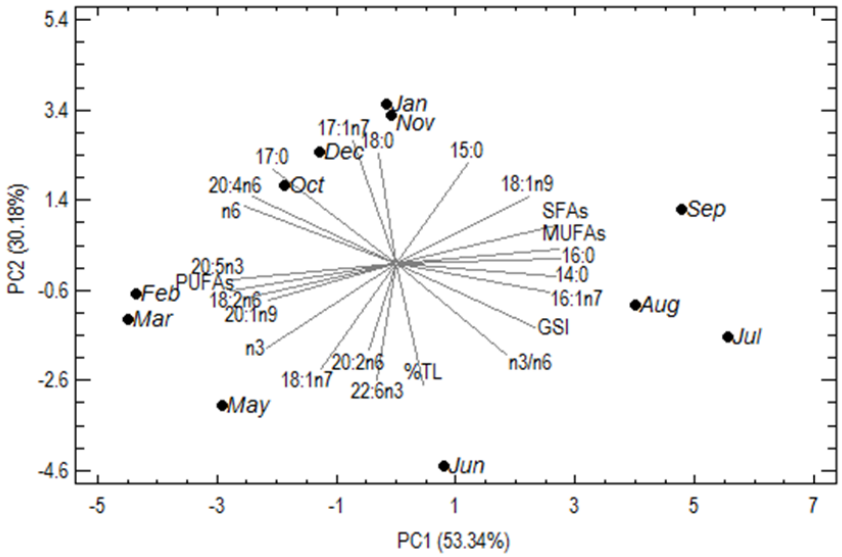

Figure 10.2. Biplot of principal component analysis (PCA): relationships between GSI, percentage of Total Lipids and FAs composition (FAs < 1% were excluded; 11 observations, 23 variables).

Table 10.1. Principal Component Analysis. Eigenvalues, explained and cumulative variance, loadings of the variables for the first three PCs.

	Principal Components		
	1	2	3
Variance explained			
Eigenvalues	12.27	6.940	1.658
% of variance	53.34	30.18	7.21
Cumulative %	53.34	83.54	90.75
Factor loadings			
GSI	**0.231**	-0.176	**0.248**
%TL	0.046	**-0.336**	-0.171
14:0	**0.266**	-0.038	0.030
15:0	0.120	**0.277**	**-0.279**
16:0	**0.273**	0.014	-0.054
16:1n-7	**0.255**	-0.079	**-0.276**
17:0	-0.205	**0.258**	-0.030
17:1n-7	-0.073	**0.338**	-0.239
18:0	-0.030	**0.305**	**0.361**
18:1n-9	0.222	0.184	0.102
18:1n-7	-0.126	**-0.292**	-0.194
18:2n-6	**-0.243**	-0.093	-0.222
20:1n-9	-0.213	-0.104	**0.271**
20:2n-6	-0.046	-0.239	**-0.483**
20:4n-6	**-0.241**	0.184	-0.004
20:5n-3	**-0.273**	-0.045	-0.041
22:6n-3	-0.033	**-0.321**	**0.265**
SFAs	**0.269**	0.104	0.048
MUFAs	**0.270**	0.038	-0.191
PUFAs	**-0.277**	-0.077	0.055
n-3/n-6	0.183	**-0.254**	0.199
n-3	-0.215	-0.236	0.062
n-6	**-0.253**	0.159	-0.039

Supplementary Table S10.1. Annual variations in FAs composition (% vs total FAs) in the ovary of *P. Kerathurus* females at different stages of ovarian development. Each value is the mean ±SD of five separate prawn samples analysed in triplicate for each. Within each row, superscript letters indicate significant differences ($p< 0.05$). On the right column the correlation index r with GSI (*$p< 0.5$; **$p<0.01$). In grey lipids below 1% of the total FAs.

Lipids	Feb	March	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	ANOVA, p value	GSI correlation r	
12:0	0.03±0.01	0.31±0.1	0.04±0.01	0.61±0.2	0.59±0.5	1.17±0.5	0.73±0.3	0.15±0.1	0.16±0.1	0.09±0.03	0.16±0.1			
14:0	0.66±0.2^{a}	1.61±0.5^{b}	0.65±0.2^{a}	2.83±0.9^{d}	4.54±0.2^{e}	5.90±0.3^{f}	4.27±0.1^{e}	1.57±0.1^{b}	2.03±0.1^{b,c}	2.32±0.2^{c,d}	2.09±0.2^{b,c}	0.0000	0.72167	*
15:0	0.89±0.3	1.06±0.3	0.93±0.1	0.96±0.3	1.09±0.1	1.30±0.7	1.56±0.3	1.48±0.3	1.62±0.1	1.19±0.9	1.54±0.1	0.2496	-0.04993	
16:0	11.8±0.8^{a,b}	11.7±0.6^{a,b}	10.9±0.7^{a}	15.7±0.3^{e}	19.5±0.7^{f}	15.6±0.7^{e}	18.6±0.6^{f}	12.8±0.6^{b,c}	15.0±1^{d,e}	13.8±0.8^{c,d}	15.0±0.9^{d,e}	0.0000	0.81022	**
17:0	2.36±0.6^{c,d}	1.78±0.2^{b,c}	2.31±0.4^{c,d}	1.14±0.2^{a,b}	1.06±0.5^{a}	1.34±0.7^{a,b}	1.52±0.1^{a,b}	2.27±0.5^{c,d}	2.60±0.1^{d}	2.42±0.1^{c,d}	2.37±0.1^{c,d}	0.0001	-0.84941	**
18:0	7.00±1.3^{c,d}	4.82±0.6^{a}	6.39±0.6^{b,c}	5.60±0.4^{a,b}	6.07±0.7^{a,b,c}	7.00±1.0^{c,d}	5.46±1.1^{a,b}	6.45±0.5^{b,c}	8.28±0.8^{d,e}	8.34±0.3^{d,e}	8.51±0.9^{e}	0.0001	-0.38955	
20:0	0.13±0.1	0.10±0.1	0.11±0.1	0.31±0.2	0.31±0.1	0.38±0.1	0.29±0.1	0.18±0.1	0.19±0.2	0.21±0.1	0.21±0.2			
21:0	0.04±0.01	0.04±0.01	0.02±0.01	0.05±0.01	0.06±0.01	0.07±0.01	0.06±0.01	0.02±0.01	0.04±0.01	0.03±0.01	0.04±0.01			
22:0	0.11±0.2	0.04±0.01	0.09±0.01	0.10±0.5	0.14±0.1	0.13±0.2	0.11±0.1	0.04±0.01	0.06±0.01	0.06±0.01	0.07±0.02			
Total SFAs	23.2±0.3^{b}	21.6±0.6^{a}	21.9±0.8^{a,b}	27.4±0.8^{d}	33.4±0.9^{f}	33.0±0.7^{f}	32.7±0.6^{f}	25.0±0.6^{c}	30.0±1^{e}	28.4±0.9^{d}	30.0±1^{e}	0.0000	0.63789	*
14:1n-5	0.02±0.01	0.03±0.01	0.02±0.01	0.11±0.2	0.26±0.2	0.43±0.2	0.30±0.1	0.03±0.01	0.04±0.01	0.02±0.01	0.07±0.01			
16:1n-7	5.12±0.7^{a}	7.66±0.4^{d}	5.11±0.9^{a}	7.95±0.5^{d}	11.4±0.4^{f}	9.45±0.4^{e}	11.6±0.8^{f}	6.54±0.3^{b,c}	7.38±0.4^{c,d}	5.17±0.4^{a}	6.24±0.8^{b}	0.0000	0.81738	**
17:1n-7	1.75±0.1^{b,c}	1.75±0.4^{b,c}	1.80±0.3^{b,c}	1.08±0.1^{a}	1.21±0.5^{a,b}	1.36±0.7^{a,b}	2.19±0.1^{c,d}	2.50±0.6^{d}	2.56±0.1^{d}	2.33±0.2^{c,d}	2.68±0.2^{d}	0.0001	-0.65259	*
18:1n-9	8.73±0.9^{a}	9.22±0.8^{a,b}	8.63±0.1^{a}	10.3±0.7^{c}	12.1±0.4^{d}	12.8±0.6^{d}	12.9±0.9^{d}	10.8±0.5^{c}	9.94±0.3^{b,c}	10.3±0.6^{c}	10.9±0.3^{c}	0.0000	0.60859	*
18:1n-7	4.54±0.7^{b,c}	5.04±1.1^{c}	4.69±0.7^{b,c}	4.25±0.3^{a,b,c}	4.03±0.8^{a,b,c}	3.71±0.2^{a,b}	3.35±0.4^{a}	3.27±0.2^{a}	3.80±0.8^{a,b}	3.46±0.1^{a}	3.31±0.6^{a}	0.0164	-0.05463	
20:1n-9	1.90±0.4	1.54±0.4	1.98±0.3	1.67±0.6	1.39±0.3	1.38±0.4	1.29±0.4	1.66±0.4	1.24±0.4	1.80±0.3	1.51±0.1	0.3139	-0.49494	
22:1n-9	0.07±0.01	0.04±0.01	0.06±0.02	0.11±0.1	0.12±0.1	0.10±0.1	0.12±0.2	0.18±0.1	0.04±0.01	0.19±0.1	0.04±0.01			
Total MUFAs	22.2±0.3^{a}	25.3±0.4^{c}	22.3±0.4^{a}	25.5±0.7^{c}	30.5±0.6^{d,e}	29.4±0.8^{d}	31.8±0.6^{e}	25.0±0.4^{b,c}	25.0±0.5^{b,c}	23.4±0.5^{a,b}	24.7±0.4^{b,c}	0.0000	0.7458	**
18:3n-3	0.16±0.3	0.26±0.1	0.17±0.2	0.56±0.1	0.28±0.2	0.35±0.2	0.28±0.1	0.23±0.2	0.20±0.1	0.15±0.1	0.18±0.2			
20:3n-3	0.17±0.2	0.16±0.1	0.16±0.1	0.34±0.1	0.21±0.1	0.22±0.1	0.15±0.1	0.14±0.1	0.13±0.1	0.13±0.1	0.13±0.1			
20:5n-3	23.6±0.4^{e}	23.7±0.5^{e}	23.3±0.4^{e}	17.7±0.7^{b}	12.9±0.6^{a}	17.1±0.5^{b}	12.4±0.5^{a}	20.5±0.7^{d}	19.0±0.4^{c}	19.8±0.7^{c,d}	19.1±1^{c}	0.0000	-0.79611	**
22:6n-3	13.9±0.3^{b}	15.6±0.7^{c}	15.9±0.4^{c}	21.6±0.7^{d}	16.3±0.3^{c}	14.0±0.3^{b}	14.2±0.7^{b}	14.4±0.3^{b}	11.8±0.8^{a}	12.6±0.5^{a}	12.0±0.3^{a}	0.0000	0.55351	
n-3 PUFAs	37.9±0.5^{f}	39.7±1.3^{g}	39.5±0.5^{g}	40.1±0.7^{g}	29.6±0.7^{b}	31.7±0.6^{c,d}	27.0±0.5^{a}	35.3±0.7^{e}	31.1±0.9^{c}	32.6±0.8^{d}	31.3±1.1^{c,d}	0.0000	-0.32474	
18:2n-6	1.29±0.4	1.37±0.1	1.21±0.6	1.20±0.2	0.69±0.3	0.81±0.6	0.83±0.1	1.08±0.5	1.18±0.4	0.98±0.5	1.05±0.2	0.5598	-0.60527	*
18:3n-6	0.03±0.01	0.08±0.01	0.03±0.01	0.13±0.1	0.09±0.01	0.10±0.03	0.08±0.01	0.08±0.01	0.07±0.01	0.05±0.01	0.08±0.01			
20:2n-6	1.16±0.4^{a}	2.36±0.5^{b}	1.18±0.4^{a}	1.43±0.3^{a}	1.22±0.2^{a}	1.17±0.5^{a}	1.22±0.3^{a}	1.07±0.2^{a}	0.97±0.3^{a}	1.20±0.1^{a}	1.02±0.2^{a}	0.0037	0.09655	
20:3n-6	0.24±0.2	0.30±0.1	0.25±0.4	0.33±0.1	0.22±0.1	0.22±0.1	0.19±0.2	0.27±0.3	0.30±0.2	0.22±0.1	0.22±0.3			
20:4n-6	13.9±0.6^{f}	9.18±0.6^{c}	13.4±0.8^{f}	3.82±0.4^{a}	4.01±0.1^{a}	3.51±0.2^{a}	5.96±0.2^{b}	12.1±0.7^{e}	11.2±0.4^{d}	13.0±0.4^{f}	11.4±0.8^{d,e}	0.0000	-0.87721	**
n-6 PUFAs	16.6±0.2^{g}	13.3±0.6^{d}	16.1±0.4^{f,g}	6.90±0.5^{b}	6.24±0.1^{a,b}	5.81±0.3^{a}	8.28±0.2^{c}	14.6±0.5^{e}	13.7±0.4^{d}	15.5±0.4^{f}	13.8±0.8^{d}	0.0000	-0.8997	**
16:2n-7	0.12±0.1	0.15±0.1	0.14±0.1	0.13±0.1	0.14±0.1	0.14±0.1	0.14±0.1	0.11±0.1	0.13±0.1	0.13±0.2	0.11±0.1			
Total PUFAs	54.7±1.6^{d}	53.1±1^{d}	55.7±1.6^{d}	47.2±1.7^{b,c}	36.0±2.4^{a}	37.6±2.7^{a}	35.5±1.5^{a}	49.9±1.3^{c}	44.9±1^{b}	48.2±0.9^{c}	45.2±1.4^{b}	0.0000	-0.72189	*
n-3/n-6	2.28±0.1^{a}	2.98±0.3^{b,c}	2.46±0.1^{a,b}	5.81±0.1^{e}	4.75±0.4^{d}	5.44±0.6^{e}	3.27±0.1^{c}	2.42±0.06^{a,b}	2.28±0.7^{a}	2.11±0.2^{a}	2.27±0.3^{a}	0.0000	0.82984	**

10.5 References

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11 Concluding remarks

During my research, I used lipids (in particular fatty acids) as biomarkers to study the influences of different variables in different marine organisms. In particular: the effect of time and temperature on Antarctic fish *Trematomus bernacchii*, the influence of size and gender on meat quality of the Atlantic Bluefin tuna, the change in lipid profile during gonad maturation of Caramote prawn *Penaeus kerathurus*.

The results showed that every change in external (environmental, diets, etc.) or internal (size, gender, biological cycles, etc.) factor corresponded to changes within the lipid profile, with a particular influence on fatty acid composition.

The study of lipid profile and the monitoring of specific fatty acids are an important aspect of the research because lipids are very important molecules in a biological system and, they have fundamental roles as reserve of energy, as building blocks of phospholipids and glycolipids (constituents of cell membranes), as precursors of messengers molecules. A specific change of internal or external factors, corresponds to variations of FA composition. By studying the effect and the mechanisms of internal and external factors on lipid profiles, it is possible to create a model of prevision. In the other way, if there is a change in lipid profile or in a specific fatty acid, knowing the general condition of the animals, then it is possible to find the cause that may have triggered the change.

In conclusion, the study of fatty acid composition or of lipid profiles are good instruments to understand the response of an organisms to an external or internal change. Furthermore, this approach can be a valuable complementary study to previous research involving biochemical and/or genetic investigations.

12 Publications during my PhD

Truzzi, C., Annibaldi, A., Antonucci, M., Scarponi, G., Illuminati, S., 2018. Gas chromatography-mass spectrometry analysis on effects of thermal shock on the fatty acid composition of the gills of the Antarctic teleost, Trematomus bernacchii. Environ. Chem. 15, 424–435. https://doi.org/https://doi.org/10.1071/EN18130

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