Universidade de Aveiro Departamento de Biologia
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Hidrocarbonetos poliaromáticos em tecidos hepáticos de *Etmopterus pusillus* e *Galeus melastomus*: análise de biomarcadores

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Virgília Sofia Almeida de Azevedo e Silva, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro e do Doutor Carlos Alexandre Sarabando Gravato, Professor Auxiliar do Departamento de Biologia Animal Da Faculdade de Ciências da Universidade de Lisboa.

o júri

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agradecimentos

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palavras-chave

Hidrocarbonetos aromáticos(PAH´s), *Etmopterus pusillus*, *Galeus melastomus*, biomarcadores, stress oxidativo.

resumo

Um dos maiores ecossistemas do planeta, o marinho, apresenta uma biodiversidade enorme e é uma fonte de diversos recursos naturais. Hoje em dia, este ecossistema tornou-se relevante devido ao facto de ser alvo de inúmeras pressões antropológicas.

Os tubarões são um dos predadores que estão no topo das cadeias alimentares marinhas, e estão constantemente a absorver contaminantes como consequência da bioacumulação nas redes tróficas. *Etmopterus pusillus* e *Galeus melastomus* no Oceano Atlântico estão a sofrer os efeitos da bioacumulação dos hidrocarbonetos poliaromáticos (PAH´s). Por essa mesma razão, no presente estudo foram avaliados um número de biomarcadores como objetivo de estabelecer uma relação entre os níveis de PAH´s e a ocorrência de stress oxidativo em elasmobrânquios.

Na pesca comercial, foram capturados 37 indivíduos de *Etmopterus pusillus* (23 machos e 14 fêmeas) e 15 indivíduos de *Galeus melastomus* (todos fêmeas) no Oceano Atlântico, nos arredores da costa de Sesimbra. O tecido hepático foi colhido e congelado a -80°C. As amostras hepáticas foram divididas em três grupos de idade: juvenis, adultos e séniores, separados de acordo com o seu peso e tamanho individual.

Neste estudo, alguns PAH´s foram quantificados como, naftaleno, pireno, fenantreno e benzo[a]pireno através do método fluorimétrico. Os PAH´s foram detetados em todas as amostras de tecido hepático dos juvenis, adultos e sénior e em ambas as espécies.

Visto que foram detetados níveis de PAH´s, diferentes indicadores de stress oxidativo foram quantificados: atividade da catálase (CAT), catividade da glutationa s-transferase (GST), glutationa total (TG), peroxidação lipídica (LPO), sistema transportador de eletrões (ETS), hidratos de carbono (HC) e lípidos.

Nos tecidos hepáticos de *Etmopterus pusillus* foi observado que CAT, GST e LPO aumentam no grupo dos adultos. Contrariamente, TG , ETS e lipidos diminuem no grupo adulto quando comparados com os juvenis. Relativamente ao *Galeus melastomus* foi observado que GST e LPO diminuem no grupo dos adultos quando comparados com os juvenis. Como a acumulação de PAH´s nos tecidos hepáticos foi detetada e foram encontradas diferenças significativas nos níveis das defesas antioxidantes, isto pode indicar uma relação entre eles, e um eventual estado de stress oxidativo desta população de elasmobrânquios.

keywords

Polyaromatic hydrocarbons (PAH´s), *Etmopterus pusillus*, *Galeus melastomus*, biomarkers, oxidative stress.

abstract

One of the biggest ecosystems on the planet, the marine ecosystem, represents huge diversity and is a source of many different natural resources. Today, this ecosystem has become relevant due to the fact that planet ecosystems are a target of several anthropogenic pressures.
Sharks are one of the top predators in marine food chain, constantly absorbing contaminants as a consequence of bioaccumulation on trophic nets. *Etmopterus pusillus* and *Galeus melastomus* in the Atlantic Ocean are suffering the effects of polyaromatic hydrocarbons (PAH´s) bioaccumulation. For that reason, in the present report were evaluated a number of different biomarkers in order to stablish the relationship between PAH´s levels and the occurrence of oxidative stress in elasmobranchs.
In commercial fishing, 37 individuals of *Etmopterus pusillus* (23 males and 14 females) and 15 individuals of *Galeus melastomus* (all females) were captured in the Atlantic Ocean, off the coast of Sesimbra. Hepathic tissue was collected and frozen at -80°C. Hepathic samples were split into three age groups: juveniles, adults and seniors, split according to the specimen's total weight and size.
In this study, several PAH´s were quantified, such as naphthalene, pyrene, phenanthrene and benzo[a]pyrene content by a fluorimetric method. PAH´s were detected in hepathic tissue samples of juveniles, adults and seniors group in both species.
Since PAH´s accumulation in liver tissue were detected, different stress oxidative parameters were quantified: catalase activity (CAT), glutathione s-transferase activity (GST), total glutathione (TG), lipid peroxidation (LPO), electron transport system (ETS), hydrocarbons (HC) and lipids.
In hepathic tissue of *Etmopterus pusillus* it was observed that CAT, GST and LPO increased in adult group. Conversely, TG, ETS and lipids decreased in adult group when compared with juvenile group. Relatively to *Galeus melastomus*, it was observed that GST and LPO decreased in adult group when compared with juvenile group. As PAH´s accumulation in hepathic tissues were detected and significant differences in antioxidant defense levels were found, it might indicate a relationship between them, and eventually an oxidative stress state of elasmobranch´s population.

ABBREVIATIONS

ATP – Adenosine Triphosphate

BHT – 2,6-Di-tert-butyl-4-methylpheno

CAT – Catalase

CDNB – 1-Chloro-2,4-dinitrobenzene

CH_3OH – Methanol

$CHCl_3$ – Chloroform

DNA – Deoxyribonucleic acid

DTNB – 5-5'-dithibiobis (2-nitrobenzoic acid)

DTPA – Diethylenetriaminepentaacetic acid

ETS – Electron Transport System

FACs – fluorescent aromatic compounds

EROD – Ethoxyresorufin-o-deethylase

GR – Glutathione reductase

GSH –L-Glutathione reduced

GST – glutathione S-transferase

H_2SO_4 – Sulfuric acid

HCl - Hydrochloric acid

INT – Iodonitrotetrazolium chloride

LPO – Lipid Peroxidation

NADH-Nicotinamide adenine dinucleotide

NADPH – ß-Nicotinamide adenine 2'-phosphate

$NaHCO_3$ – Sodium Bicarbonate

MO – Microsomal Monooxygenase

MOs – Membrane bound proteins

MFO – Mixed Function Oxidase

PAH – Polycyclic Aromatic Hydrocarbon

PAH´S – Polyaromatic Hydrocarbons

PMS – Post-Mitochondrial Supernatant

ROS – Reactive oxygen species

TBA – 2-Thiobarbituric acid

TCA – Trichloroacetic acid

TG – Total Glutathione

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I- INTRODUTION

1. Marine Pollution

One of the biggest planet ecosystems, the marine presents a huge diversity and it's a source of different natural resources. Nowadays, it´s become relevant the fact that planet ecosystems are a target of several anthropogenic pressures. Several times are used as garbage reservoir from human activity, and it came obviously, that reach preoccupant levels in parameters that translate the water quality (de la Torre *et al*., 2007). Since the seventies, it has been clear a huge industrialization index and an over exploration of the natural resources in addition with a crescent consumption per habitant and the need to produce more and more every day, the ecosystems are constantly over pressure.

2. Xenobiotics

Xenobiotics include several chemical compounds as animal toxins, natural hydrocarbons and plant products and his complexity range from small inorganic ions until large organic molecules. Regardless the capacity of environment to absorb and degrade the pollutants some of them are more persistent and could induce biological damages in aquatic organisms.

Polyaromatic hydrocarbons (PAH´s) are planar molecules with variable number of benzene rings (two or more) and have genotoxic and carcinogenic properties, thermodynamically stable with high melting and boiling points (Doyle *et al*., 2008). They are formed during the incomplete combustion of fossil fuels and are known over 100 different molecules with 16 in the list of priority pollutants (all the xenobiotics quantified in this thesis are in this list) (Achten *et al*., 2011; Vidolova, 2014) (Figure 1, Table I). Regarding chemical properties, PAH´s can be divided in two groups: low molecular weight (compounds compose until 3 aromatic rings) and high molecular weight (containing four or more aromatic rings) (Doyle *et al*., 2008).

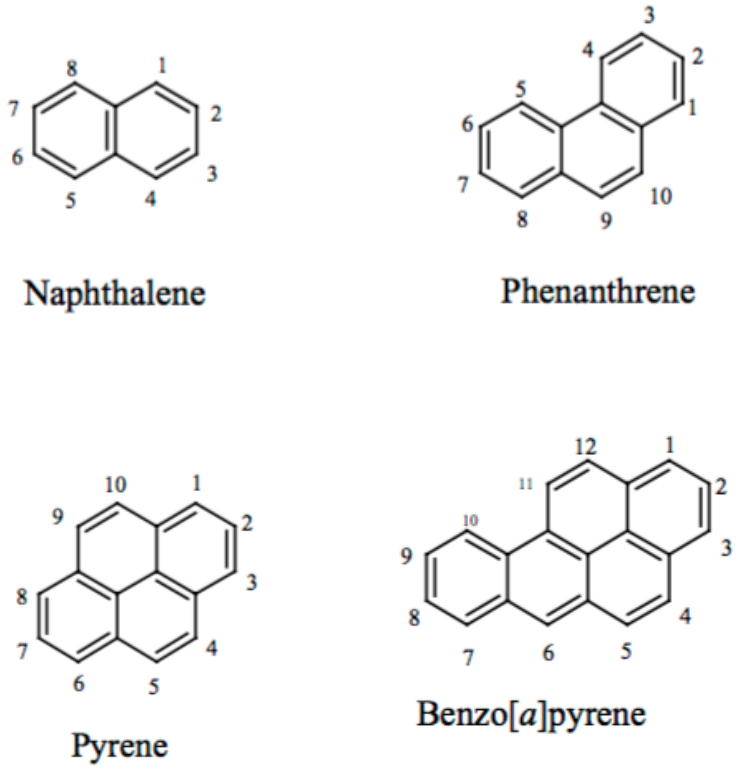

Figure 1-Representation of the molecular structure of PAHs quantified in this work.

Hydrocarbons of anthropogenic origin are distributed in different ecosystems, i.e., water, soils and biota and the quantification of polyaromatic hydrocarbons (PAH´s) in aquatic animals and sediments are a measure to identify the quality of the environment (Nowack & Bucheli, 2007). The impacts of hydrocarbons at cellular levels are very important since is a measure to control quality in marine environment and the fitness of marine organisms (Notar *et al*., 2001).

One example of consequences of contaminants occur with *Paleamon serratus*, a benthic specie which absorbed contaminants from waters and sediments causing swimming difficulties and remaining bioavailable for several years (Gerhardt *et al.*, 2002). Seabass expose to benzo[a]pyrene have shown higher sensitivity, and as consequence erithrocytic genotoxic effects were reported (Gravato & Guilhermino, 2009). Earlier effects of pollutants on cellular level were observed, allowing the development of biomarkers to control the changes in the metabolism and prevent a major impact (Lemos *et al*., 2010; Alves *et al*., 2016).

It became a priority, find out the consequences and effects of several xenobiotics that are in contact all the time with different animals, which in turn has a real impact in top predator's metabolism, like sharks (Rudneva, 1999).

Due to the permanent contact with oxygen, the metabolism of aerobic animal produce and eliminate reactive oxygen species (ROS) (O^{2-}, H_2O_2, •OH) in a concentration balance ("steady-state levels") (Lushchak, 2011a). As the name itself indicates, this molecules are extremely reactive and if are not eliminate could damage certain cellular and metabolic processes (Lushchak, 2011b; Mountouris *et al*., 2002; Storey, 1996).

When the "steady-state level" is unbalanced, the organism is in "oxidative stress". The two types of cellular defence for fight oxidative stress are: I. regulation of membrane permeability and II. Antioxidant potential (Lushchak, 2011a) (Figure 2).

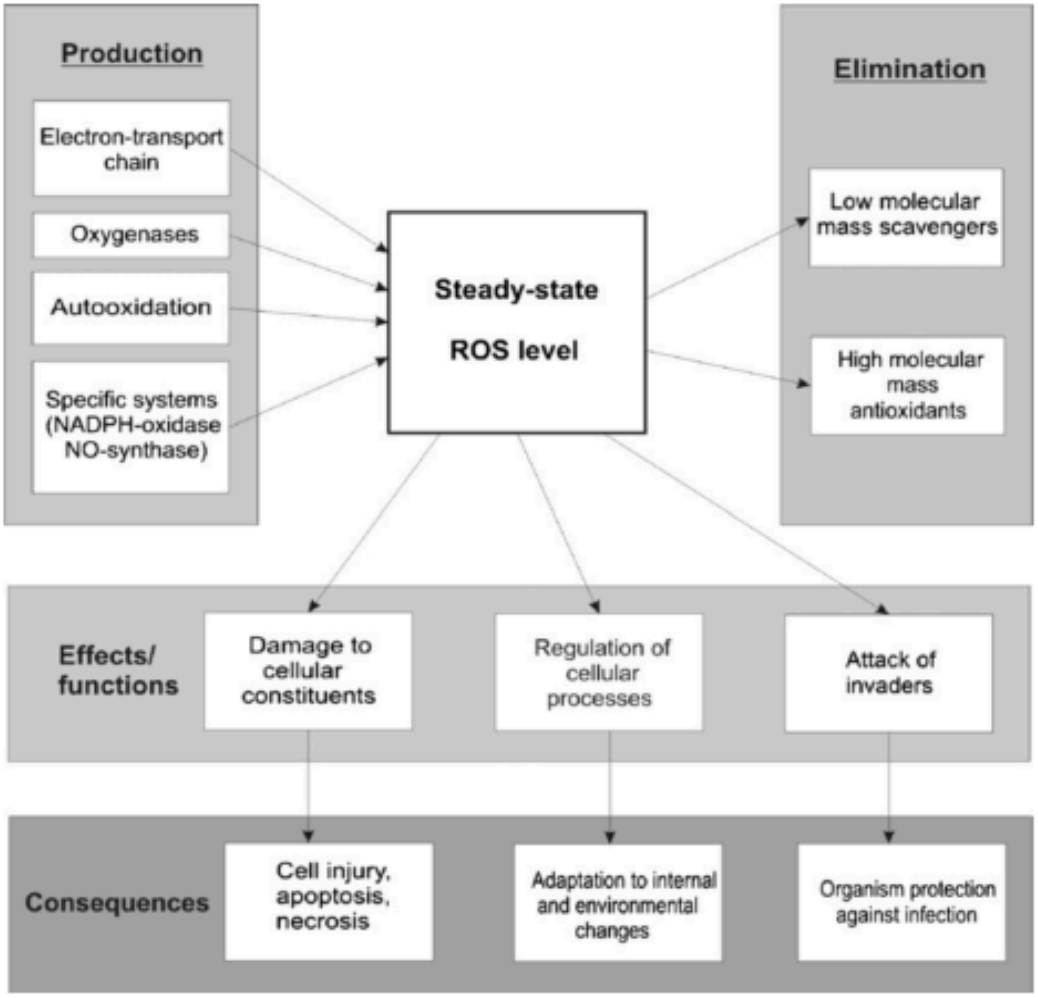

Figure 2-Schematic relationship between production and elimination of ROS level. Integral Image from "Environmentally induced oxidative stress in aquatic animals"; Lushchak, 2010.

When an individual is in an "oxidative stress state" several modifications in the metabolism occur: I. oxidation of proteins, DNA and steroid components and II. peroxidation of unsatured lipids in cell membranes. The second occurrence threats the cellular integrity because produce unstable and reactive lipid hydroperoxides (Martínez-Álvarez, Morales & Sanz, 2005).

In general, defensive strategies to prevent O_2 toxicity in the cellular medium are more complex and diverse according bigger complexity of individuals. Antioxidant defences are the first in counteract the biological oxidation, like vitamin C, uric acid, glutathione and carotenoids. Together with antioxidant enzymes

(catalase, glutathione S-transferase (GST) and superoxide dismutase) prevent the oxidant reactions and closing the lipid-peroxidation cycle (Martínez-Álvarez *et al*., 2005).

For the organism protection to ROS exist two types of defences: antioxidants enzymatic and non-enzymatic (Storey, 1996). Glutathione S-transferase it´s very important in the second phase of xenobiotics elimination (Valko *et al*., 2007) (Figure 2).

The presence of contaminants, like heavy metals and PAH´s, increase the levels of ROS species such hydrogen peroxide (H_2O_2). The presence of oxidizing molecules promotes activation of enzymatic antioxidant defences, i.e., catalase (CAT) (EC 1.11.1.6), glutathione S-transferase (GST) (EC 2.5.1.18). Besides contaminants, there´s a few factors that induce oxidative stress, i.e., temperature, oxygen level, salinity, transition metal ions (iron and chromium) (Lushchak, 2011b).

3. Biotransformation

Marine species are constantly absorbing through permanent exposure (mostly by gills and skin) to xenobiotics being necessary the excretion of pollutants. By biotransformation the compounds are transformed, in liver, into several hydrophilic products more easily excreted (Vermeulen, 1996).

Detoxification can be divided in two phases: Phase 1 and Phase 2. Phase 1 consist in oxidation, reduction and hydrolysis and it can be catalysed for cytochrome P450 or Mixed Function Oxidase (MFO) increasing the compound electrophilic characteristic allowing the compound to be processed in phase 2. In the next phase a polar endogenous group (e.g. glutathione) is conjugated to phase 1 metabolite to increase the capacity to excrete the compounds (Wassmur, 2012).

MFO is constituted for microsomal monooxygenase (MO) enzyme that have a great capability for organic xenobiotics degradation and normally is located in liver (Stegeman, 2000). In oxidative biotransformation on phase 1, cytochrome P450-dependent MOs (membrane bound proteins) is the most common catalytic pathway and is located on liver endoplasmic reticulum (Wassmur, 2012).

4. Biomarkers

Face the current discharge of anthropogenic pollution at marine ecosystems, it´s a priority predicts changes in aquatic environment as well as in organisms, through certain environments, physiological and cellular parameters (Hook *et al*., 2014).

Biomarkers can be into two major groups: biomarkers of health effects from contaminant exposures and biomarkers of exposure to environmental stressors. Exposure biomarkers must show an early response to certain class of contaminants and could incorporate in this typology the following biomarkers: bile fluorescent aromatic compounds (FACs), cytochrome P4501A mRNA or protein, Ethoxyresorufin-o-deethylase (EROD) and metallothioneins. Biomarkers of effects as an indicator of physiological or biochemical changes can indicate some type of alteration in organism fitness. Can be direct measures (DNA damage) and indirect measures (impact on sub-cellular lysosomes) (Hook *et al*., 2014).

Biomarkers also could be divided in hierarchical levels: metabolite, biomolecular, organelle, cellular, tissue, organ system and organism (Walker et al., 2012). Lower levels, as biomolecular and organelle, can be an early-warning for higher levels damage and a warning for a bigger damage if the xenobiotics exposure keep going (Walker *et al.,* 2012).

Biomarkers for oxidative stress have an optimal characteristic's to be used in ecotoxicology: I. low cost and simple procedure, II. responsiveness, III. sensitive to a several parameters, IV. can tested innumerous conditions (Nunes *et al*., 2008)

Biomarker was defined as "*measuring parameters providing the means to reflect the interaction between a particular biological structure and stressful factors found in the environment under consideration, which may be chemical, physical or biological*" (de la Torre *et al*., 2007).

In the context of this thesis, our stressful factor is the presence of PAH´s on shark´s liver and the parameters are some enzymatic and non-enzymatic antioxidants defences.

Antioxidants defences, like glutathione S-transferase (GST) and catalase (CAT) are perfect biomarkers to detect oxidative stress because they inhibit formation of free radicals (Lopez-Torres, Perez-Campo, Cadenas, Rojas, & Barja, 1993). Other, like lipid peroxidation (LPO) give us information about the effect of ROS in biological lipids. Lipid peroxidation is related to membrane-bound enzymes destabilization (Solé *et al*., 2008).

In general, fish are used as environment and pollution bioindicator (Farombi *et al*., 2007). Basically, marine population are in contact with several contaminants and absorb them through ingestion, inhalation, dermal and branchial contact (Vidolova, 2014).

Marine population is susceptible to reactive oxygen species and develop several defences, mostly adapted enzymes like catalase, superoxide dismutase and enzymes dependent on glutathione. Other substances are present in antioxidant defences like amino acids, vitamin E, C and K and peptides (Martínez-Álvarez *et al*., 2005).

Among marine animals, sharks are in the top of several trophic relationships and food webs and can be predators or preys, live in different places of the water column from epipelagic to deep-sea benthic environments *(Xavier et al*., 2012). Sharks are a perfect population for study the impacts of pollutants on marine ecosystems because they are in the top food webs and are a perfect example for the study and quantify the consequences of bioaccumulation and biomagnification on humans (Alves *et al*., 2016).

Through an extensive analysis of alimentary habits from sharks, we can understand that higher levels of xenobiotics (heavy metals, polycyclic aromatic hydrocarbons) are found in their metabolism and could induce cellular damage and other metabolic disorders. (Wudiilf *et al*., 2007). Several studies demonstrate cellular and metabolic damages in sharks when exposed to contaminants (Alves *et al.*, 2016; Nam *et al*., 2010). Alves and collaborators (2016) reported that certain contaminants inhibit antioxidant enzymes, like GST, and affect directly biological aspects like swimming and feeding.

Nam *et al.*, (2010) demonstrate that in lemon shark (*Negaparion brevirostris*) when brain is exposed to certain contaminants, in this case brevetoxins, causes

several changes in neurochemistry as functional damage in neurochemistry and sub-clinical effects. Concluded that the effects that causes in sharks is dependent of age, gender and weight.

Elasmobranchs, has scientific interest, since is an ancient group, with particular evolutionary position specific strategies for osmosis and a lower index of neoplasia (Laganà *et al.*, 2006). Two different orders of Elasmobranchii: Carchariniformes and Squaliformes were studied in this thesis, *Galeus melastomus* and *Etmopterus pussilus,* respectively both deep-water sharks (Laganà *et al*., 2006).

G. melastomus (black mouth catshark) is a shark with small dimensions, lives in the eastern Atlantic and Mediterranean Sea Basin (Anastasopoulou *et al.*, 2013). Opportunist predator, habits between 300 and 1600 meters deep and has a diet based on three big groups: fishes, cephalopods, shrimps and other crustaceans (Valls *et al*., 2011; Anastasopoulou *et al*., 2013).

Due to his low commercial price, *E. pussilus* (common name - lantern shark) is not reported frequently and his ecology, biology and physiology are barely known. Is a deep water lantern shark with a global distribution, found at depths between about 300 and 1000 m (Xavier *et al*., 2012). His diet is composed by mesopelagic and demersal crustaceans, fish and cephalopods. *E. pussilus* fed across the water column that implies a great diversity in his diet , and eventually can be a prey instead of a predator and be hunt by other sharks like, *Galeus melastomus* and *Etmopterus spinax* (Xavier *et al*., 2012).

5.Objectives of the thesis

The objective of this work is to understand the liver PAH's levels and the oxidative stress. With this purpose, it was quantified the levels of PAH´s in liver of elasmobranchs and different parameters (biomarkers) related with sub-cellular metabolism, like, catalase, glutathione s-transferase, lipid peroxidation. Analyse the organism “state” by the determination of ETS and energetic reserves. This study will allow us to understand the levels of polyaromatic hydrocarbons and antioxidant defences of two species of elasmobranchs, *Etmopterus pusillus* and *Galeus melastomus*.

II- MATERIALS AND METHODS

Samples

Galeus melastomus (blackmouth catshark*)* and *Etmopterus pusilus* (smooth lantershark) were capture during the months of April and May, near Sesimbra (Centre of Portugal) in Atlantic Ocean by commercial fishing (Figure 2). Immediately after, parametric measures (weight and size) were taken (see Table II and III), shark's samples were stored in pollution-free sealed polythrene and transported to the CESAM laboratory and stored at -80 ºC.

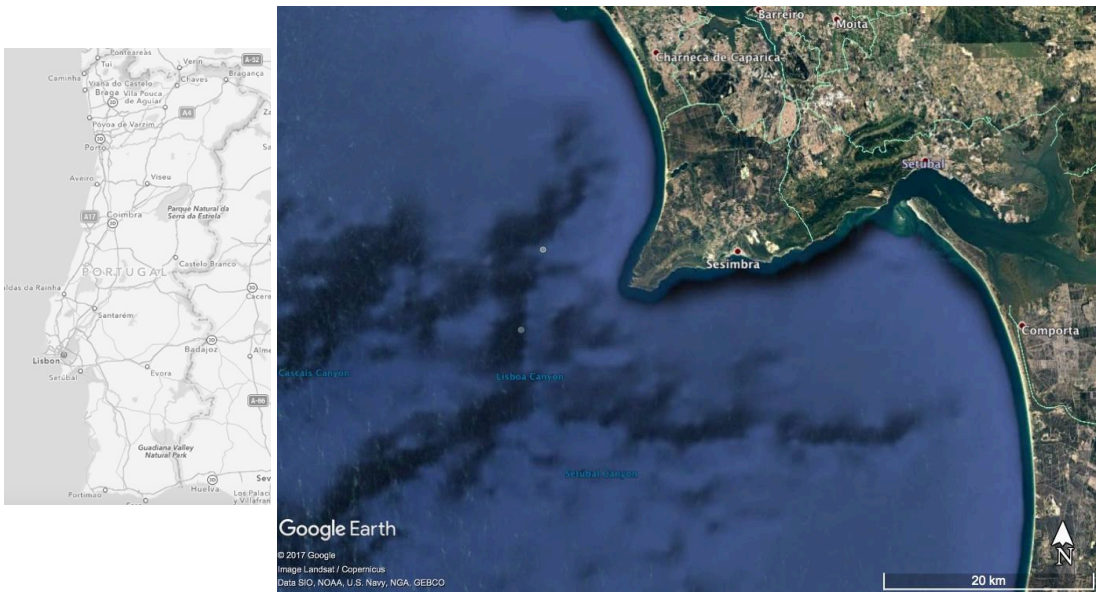

Figure 3-Map of the sampling area, in the center of Portugal, near Sesimbra (Source: Google Earth).

Samples Processing

After unfreezing the hepathic samples, a liver portion between 0.18 g and 0.25 g was cut and kept in ice. Each sample was homogenized with ice-cold ultra-pure water in a proportion of 1 g per 10 mL, by using a sonic homogenizer (10 - 20 seconds).

The obtained homogenate was separated in aliquots to perform different biomarkers assays: 300 µL for electron transport system (ETS), 300 µL for lipid, 300 µL for carbohydrates and reserve proteins quantification, 200 µL to determine the

extent of endogenous lipid peroxidation (LPO) with 4 µL of butylated hydroxytoluene (BHT) (to prevent artificial lipid oxidation) and 800 µL to isolate a post-mitochondrial supernatant (PMS) fraction with 800 µL K-phosphate buffer (0.2 M; pH = 7.4).

To isolate the PMS, the tissue homogenate (1600 µL of total volume) was centrifuged at 9,000x*g* for 20 min. at 4°C. The PMS fraction obtained was aliquoted to perform distinct quantification assays: 250 µL for total glutathione (TG), 250 µL for glutathione S-transferase (GST), 50 µL for catalase (CAT), 50 µL for protein and 50 µL for polyaromatic hydrocarbons (PAH´s). All aliquots were stored at -80°C until use.

Estimation of Polyaromatic Hydrocarbons (PAH´s)

Polycyclic aromatic hydrocarbons (PAH´s) are distributed in different environmental compartments. Specifically in marine environments, levels of PAH´s detected can indicate an extreme pollution situation in the water, near coast or in the sediment (Neff, 1985).

PAH´s quantified are homologues of naphthalene, phenantrene, pyrene and benzo[a]pyrene (Figure 2). To quantify these compounds an easy and cheap method – fluorometric method, was used. Basically, this method measures the fluorescence intensity when is emitted a radiation in a certain wavelength. With this objective, the PMS aliquots were unfrozen and 1450 µL of methanol 50% was added to each one. Then samples were sonicated during 1 min. at 25ºC. All the samples were quantified for the 4 homologues of PAH's (naphthalene, phenanthrene, pyrene and benzo(a)pyrene) using a HITACHI F-700 fluorimeter. Fluorescent readings were performed for naphthalene-type homologues at an excitation/emission 290/335 nm, benzo(a)pyrene-type homologues readings were made at 380/430 nm, for pyrene-type homologues readings were made at 341/383 and for phenanthrene-type homologues readings were made at 256/380 nm. The results were expressed as µg/mg of wet tissue. In this study, homologous of PAH´s are named by PAH´s.

Estimation of Biomarker´s

Nowadays, we have several types of biomarkers with different proposals, but in this work, we choose biomarkers at sub-cellular level, homologous of PAH´s,

biochemical biomarkers, and oxidative stress markers. A fastest and not very expensive method to quantify biomarkers activity and levels is the microplate´s. A non-sterile microplate is used and the absorbance emitted from sample at pretend wavelengths was measure. The only instrument needed is a microplate reader and a 96-well plate.

Catalase activity

Catalase (EC 1.11.1.6) is one of the most abundant in the cellular medium, composed by four chains and it is related with age. The main function is the defence against ROS and convert hydrogen peroxide into water and oxygen gas (Chelikani, et al., 2004).

CAT activity in PMS fraction was determined by measuring the H_2O_2 consumption at 240 nm (Clairborne, 1985). To quantify the activity of catalase a hydrogen peroxide solution was need: 37.2 µL of H_2O_2 mixed with 15.0 mL of K-phosphate buffer (0.05 M, pH=7.0). Briefly, 140 µL of K-phosphate buffer (0.05 M, pH 7.0) and 150 µL H_2O_2 50 mM were added to 10 µL of liver PMS and the enzymatic activity was determined in a spectrophotometer (Thermo Scientific Multiskan Spectrum) during 2 min. (each 20 sec) at 25°C. The results were expressed in µmol/min/mg protein and quantify the amount of enzyme required to reduce 1 µmol of H_2O_2.

Total glutathione (TG)

Glutathione has an important role in the cellular antioxidant defences and in the regulation of metabolic pathways (Lushchak, 2011b). Antioxidant and detoxifying agent is involved in the formation of disulphide bonds. Reacts with some dangerous chemical species, such as free radicals and forms inactive products. The fastest oxidation of glutathione derivate from SH group (cysteine) confers several metabolic functions to redox pair GSH/GSSG, e.g., metabolic ions regulation, cysteine transport and store, prevents oxidation on molecules, radicals and ROS reduction (Silva, 2008). Has a role in the amino acid transport across the cell membrane (Wu *et al.*, 2004).

For assessing total glutathione, 250 µL of reaction buffer with 6 mL DTNB, 3 mL NADPH (5-5'-dithibiobis (2-nitrobenzoic acid)) and 1.5 mL glutathione reductase

(GR) in 18 mL Na-K phosphate buffer (0.2 M; pH= 8.0) and 50 µL of sample, and glutathione content was monitored at 412 nm for 3 min (Rahman *et al*., 2007) GSH was quantified from calibration curve using either GSH as standard (10 - 10000 µM) and normalized for total protein content. Protein determination in all the samples followed Bradford technique (Bradford, 1976) in triplicate, using bovine γ-globulins (Sigma-Adrich, USA) as standard. The results were expressed in nM/mg of protein and quantify the total content of glutathione (GSH/GSSG).

Glutathione S-transferase (GST)

Present in cytosol of the cells, GST are a family of enzymes that transport organic-ion in the liver (Bengal, 1981; Srivastava et al., 1982). Glutathione S-transferase (GST) (E.C. 2.5.1.18) catalyse the reduce form of glutathione (GSH) to xenobiotic substrates with the goal of detoxification (Bengal, 1981). GST isoenzymes are found in diverse organisms, such as microorganisms, fish, mammals and insects (Bengal, 1981).

GST activity was quantified by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitro-benzene (CDNB) at 340 nm (Habig et al., 1974) adapted to microplate (Frasco *et al.*, 2002). Briefly, 0.25 ml of the reaction solution (24.75 mL of phosphate buffer 0.2 M pH 6.5, 0.75 mL of 60 mM CDNB in ethanol and 4.5 mL of 10 mM GSH in ultra-pure water) was added to 200 µL of liver PMS previously diluted in homogenisation buffer in order to have a final protein concentration of 0.5 mg/mL (four replicates per sample). The optical density was measured at 340 nm in a microplate reader (Thermo Scientific Multiskan Spectrum) during 5 min at 25°C. The results were expressed as nmol/min/mg of protein.

Lipid peroxidation (LPO)

Along the years, several functions and mechanisms from LPO has been studied and discovered. LPO interfered in biological signalling and diseases in several organism communities (Niki *et al*., 2005). Lipids oxidation may occur through three different mechanisms: (i) enzymatic or non-enzymatic oxidation, (ii) free

radical-mediated oxidation and (iii) non-enzymatic, non-radical oxidation (Niki *et al*., 2005).

LPO was determined by measuring the thiobarbituric acid reactive species (TBARS) (Ohkawa *et al*., 1979), preventing lipid oxidation by adding 0.2 mM butylhydroxytoluene (Torres *et al*., 2002): briefly, 100 µL of 12% trichloroacetic acid, Tris–HCl pH 7.4 with DTPA solution and 1 ml of 0.73% thiobarbituric acid were added to 0.200 mL of liver homogenate; after an incubation at 100°C during 60 min, the solution was centrifuged at 12,000×g during 5 min, and LPO levels were determined in the resultant supernatant at 535 nm in Thermo Scientific Multiskan Spectrum microplate reader. The samples were maintained in the dark and divided in 4 replicates (each one with 100 µL) and the blanks were prepared (100 µL of homogenization ultra-pure water mixed with 4 µL of 4% BHT). Results were expressed as nmol of TBARS/g of wet tissue, using $\varepsilon=1.56\times10^{5}\ M^{-1}\ cm^{-1}$.

Cellular energy allocation

Electron transport to higher until lower levels occur in the cells and allow the production of energy. At each level it´s necessary a particular electron carrier with specific energy (redox potential). This transport produce/release energy that can be used to produce adenosine tri-phosphate (ATP) in mitochondria and it is used later for the metabolic cellular process.

ETS activity was measured on 300 µL of homogenate following a protocol adapted from De Coen and Janssen (1997) with slight modifications (Rodrigues *et al.*, 2015). To homogenate samples, 150 µL of homogenization buffer (0.3 M Tris base; 0.6% (v/v) Triton X-100 at a pH of 8.5) were added. Fifty microliters of supernatant and 150 µL of buffered solution (0.13 M Tris base containing 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 mM NADPH); and 100 µL of INT solution (*p*-iodonitrotetrazolium) 8 mM were added to a microplate. The absorbance was measured kinetically over a 3 min period at 490 nm. The cellular oxygen consumption rate conversion was performed based on the stoichiometric relationship, whereby for every 2 µmol of formazan formed, 1 µmol of oxygen is consumed. The formula of Beer-Lambert was then applied to quantify the oxygen

consumed: $A=\varepsilon \times l \times c$ (A=absorbance; ε for INT-formazan=15,900 M^{-1} cm^{-1}; l=0.9 cm; and c=oxygen consumed). Results were expressed in mJ/hour/mg wet tissue.

Energy available (sugars, lipids and proteins) was quantified following the method described by De Coen and Janssen (1997) with minor modifications for microplate reading in the Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA).

For lipids quantification, 300 µL of the homogenate samples, 500 µL of chloroform (119.38 M; ACS spectrophotometric grade, ≥ 99.8%) and methanol (32.04 M; ACS reagent, ≥ 99.8%) were added, to separate the total lipid content by centrifugation (3500 rpm for 5 min). The organic phase of each sample (100 µL) was transferred to a clean glass tube, where 500 µL of H_2SO_4 was added, these were then incubated at 200ºC for 15 min. Samples were cooled down to room temperature, and ultra-pure water (1500 mL) was added to each tube and put in a 96-well plate (Fisher Scientific) 200 µL each well (4 replicates) per hepathic sample. The absorbance of samples and tripalmitine, as a standard, were measured at 375 nm. The results were presented as J/mg of wet tissue.

Carbohydrate reserves were quantified by first adding 100 µL of 15% TCA to 300 µL homogenized samples, following an incubation of 10 min at -20ºC. After centrifugation at 1,000*x*g for 15 min at 4ºC, the supernatant was collected and used for carbohydrate measurements. With that purpose 200 µL of sample, 200 µL of phenol and 800 µL of H_2SO_4 were mixed. The pellet was then resuspended in 500 µL of NaOH, incubated for 30 min at 60ºC and neutralized in 280 µL of HCl. This fraction was used for protein measurements. To quantify carbohydrate content, standards of glucose and samples were incubated at 20ºC during 30 min and the absorbance was read on the microplate at 492 nm. Results were presented in J/g wet tissue. Total protein content was quantified following Bradford's method (Bradford, 1976). In a 96 multi-well plate, 270 µL of Bradford reagent was added to 30 µL of sample and incubated during 30 min. Absorbance was read at 592 nm and the results were expressed as J/mg of wet tissue.

Statistical Analysis

For each parameter, were performed comparisons with t-test between juvenile and adult group in order to identify statistically significant differences, for $p<0.05$. Senior group was not included in statistical analysis because it has only 2 specimens (N=2), however in figures it represented as a mean of the 2 samples.

III- RESULTS

The results include morphometric measurements, PAH´s levels, activity of enzymatic and levels of non-enzymatic antioxidants defences and quantification of energetic reserves.

Morphometric measures

Morphometric measures results contemplate two different species of Elasmobranchs: *Etmopeterus pusillus* and *Galeus melastomus.* The total number of samples is 52, being 15 females and 23 males of *E. pusillus* and 15 females of *G. melastomus* (Table II and III).

In each group, the specimens were divided per species, gender, weight and length (Table IV). For estimate samples age use the gaps in graphical analysis between total weights (g) and total length (cm) to line off the different age groups. (Figure 4 and 5).

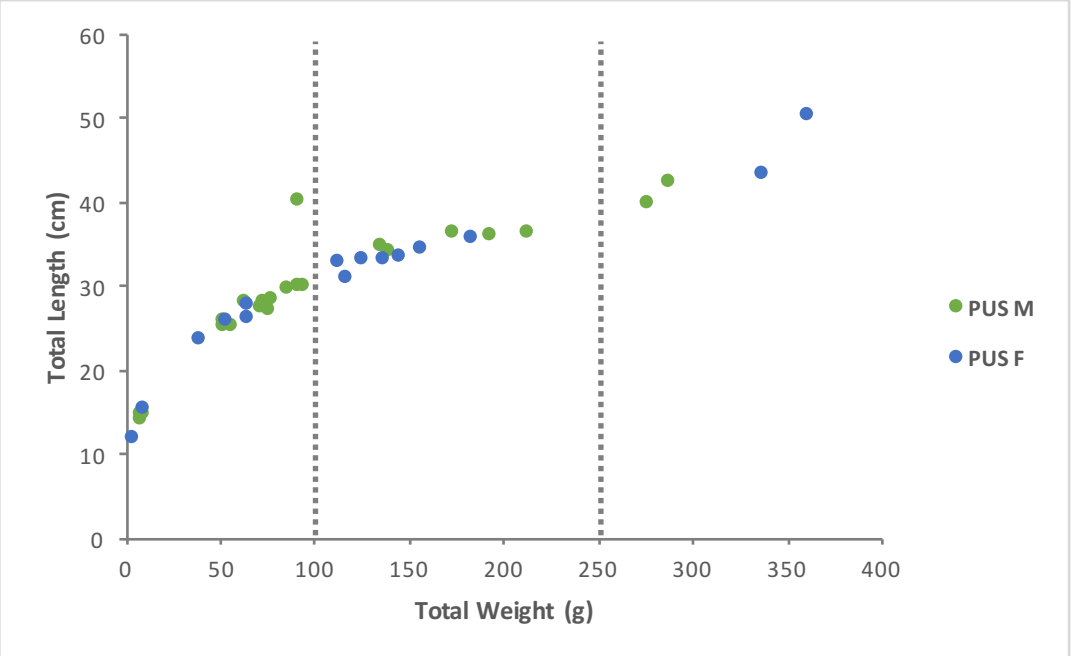

Figure 4-Male (N= 23) and female (N=14) weight (g) and length (cm) distribution of *Etmopterus pusillus*.

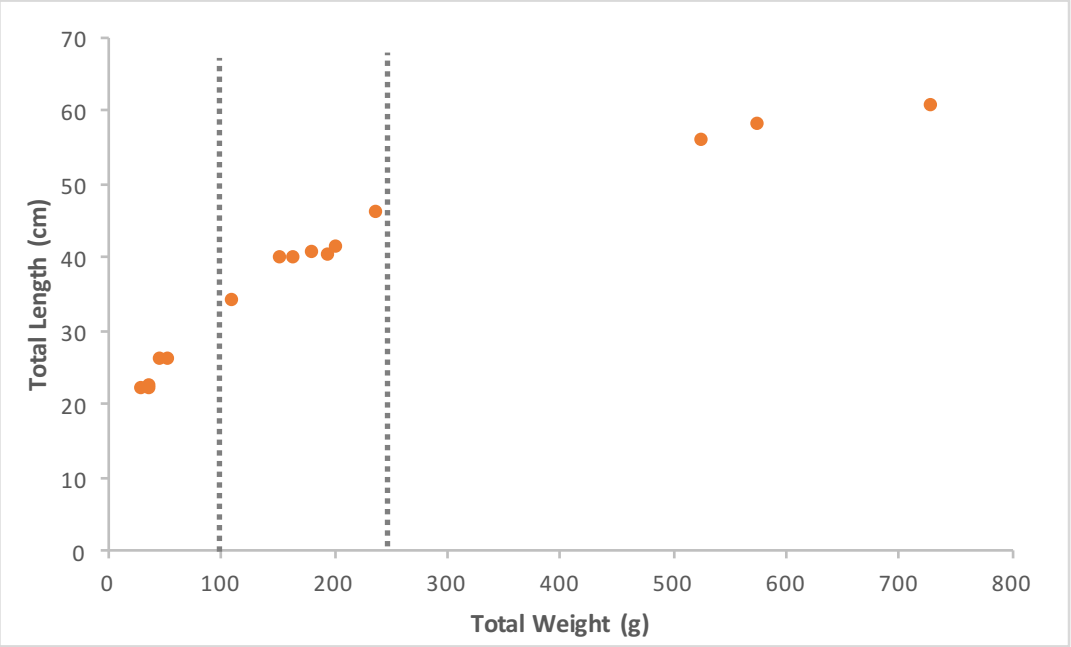

Figure 5- *Galeus melastomus* weight (g) and length (cm) distribution (only females) (N=15).

Specie	Gender	N	Juvenile (TW <100 gr)	Adult (100 gr < TW < 250 gr)	Senior (TW > 250 gr)
Etmopterus pussilus	Female	15	5	6	2
	Male	22	16	5	2
Galeus Melastomus	Female	15	5	7	3

Table IV - Total hepathic samples of *Etmopterus pusillus* and *Galeus melastomus* capture by commercial fishing, divided per individual total weight (g) in age groups (juveniles, adults and seniors).

Contaminant levels

The following contaminants quantification express the levels of four different polyaromatic hydrocarbons: naphthalene, phenantrene, pyrene and benzo[a]pyrene. They are all present in the Atlantic Ocean and have carcinogenic and genotoxic characteristics. Calibration curves were performed for each homologous PAH´s. This quantification expresses the homologous PAH´S in hepathic tissues and are expressed per gender and species (Figure 6, 7 and 8).

Naphthalene, present in pesticides and herbicides, is constituted by two aromatic rings and is the most abundant and common contaminant quantified in liver tissues.

Three benzene rings fused are in the constitution of phenantrene ($C_{14}H_{10}$) and is frequently found in tar of cigarettes. The concentration of phenantrene is the second more abundant in this study. By the other side, pyrene and benzo[a]pyrene, with carcinogenic properties, are present in liver of both species with lower concentrations than naphthalene and phenantrene.

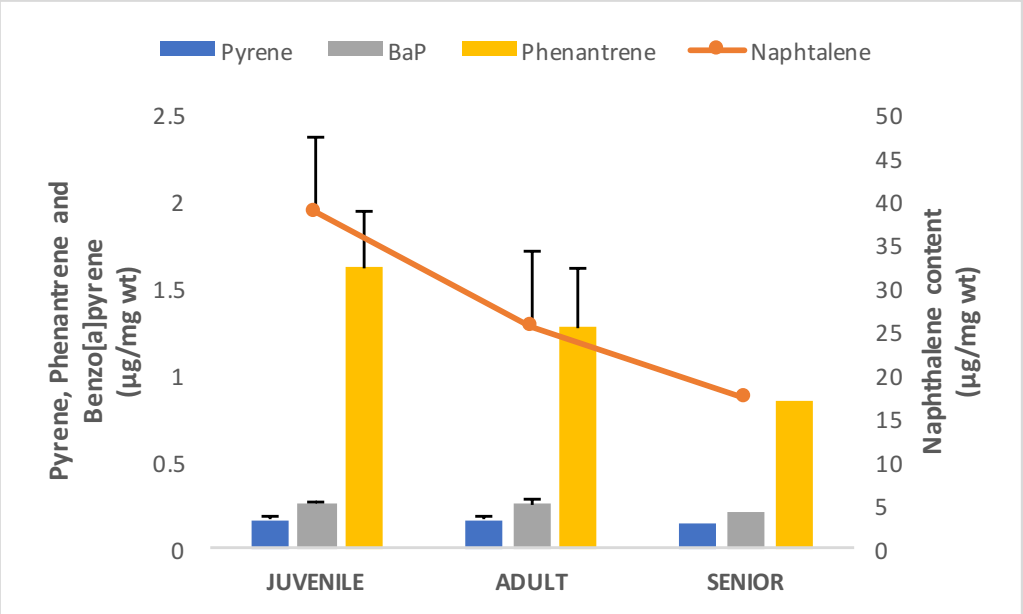

Figure 6- Polyaromatic hydrocarbons content in females of *Etmopterus pusillus* in three different ages: juvenile (N=5), adult (N=6) and senior (N=2). Naphthalene, phenantrene, pyrene and benzo[a]pyrene levels are expressed in µg/ mg of wet tissue (mean ± SEM). Senior group was calculated by the mean of two samples.

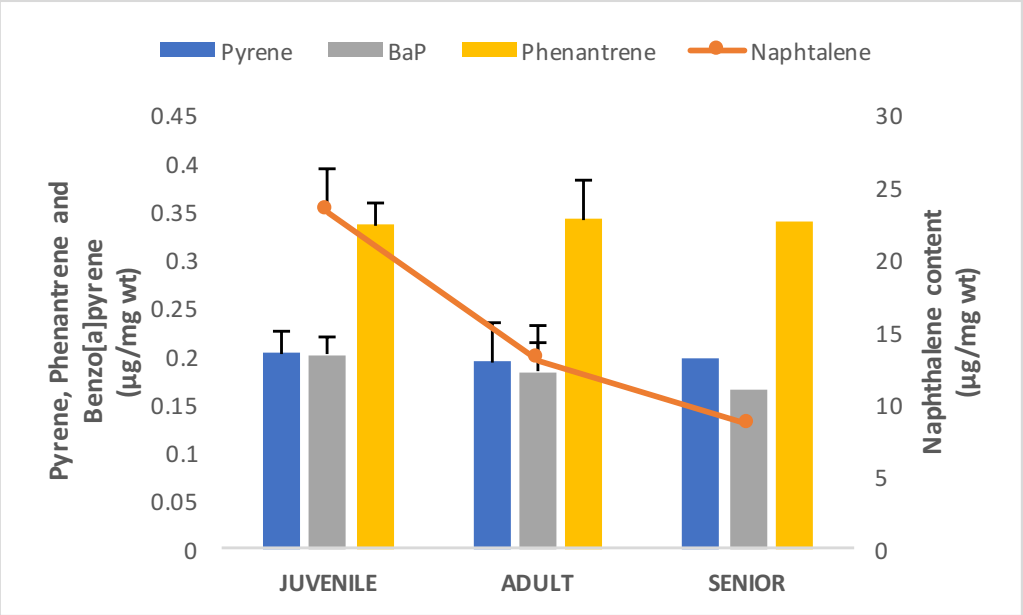

Figure 7-Polyaromatic hydrocarbons content in males of *Etmopterus pusillus* in three different ages: juvenile (N=16), adult (N=5) and senior(N=2). Naphthalene, phenantrene, pyrene and benzo[a]pyrene levels are expressed in µg/ mg of wet tissue (mean ± SEM). Senior group was calculated by the mean of two samples.

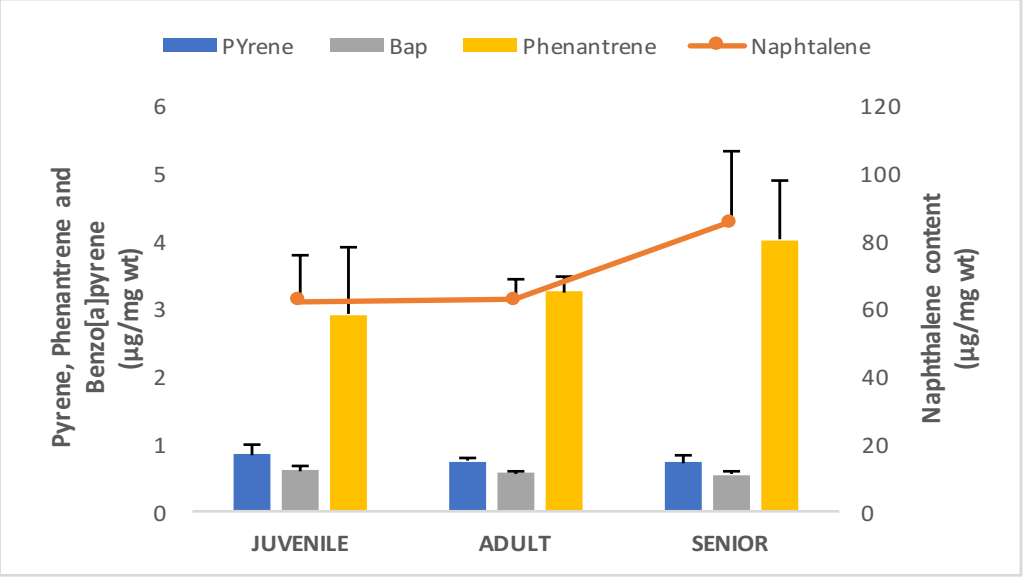

Figure 8-Polyaromatic hydrocarbons content in females of *Galeus melastomus* in three different ages: juvenile (N=5), adult(N=7) and senior (N=3). Naphthalene, phenantrene, pyrene and benzo[a]pyrene levels are expressed in µg/ mg of wet tissue (mean ± SEM).

Etmopterus pusillus

Enzymatic Antioxidants defenses

Catalase (CAT) and Glutathione S-Transferase (GST)

Figure 9 and 10 show the CAT and GST activity in PMS samples of males and females of *E. pusillus*. Catalase activity of adult females was significantly higher ($p<0.05$) when compared with juveniles and was similar between genders. Catalase activity in males increase with aging, was higher than females but was similar (Figure 9).

GST activity was similar ($p>0.05$) in adult and juveniles of both genders (Figure 10).

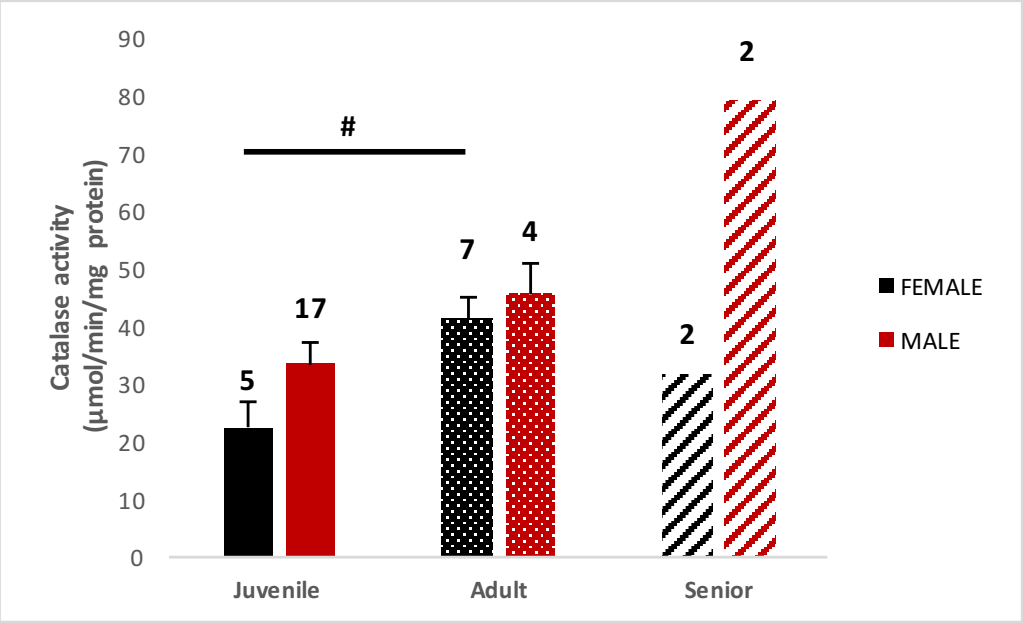

Figure 9- Comparative analysis of catalase activity in liver tissues of male (N=23) and female (N=14) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). Catalase activity is expressed in µmol/min/mg of protein (mean ± SEM). Senior group was calculated by the mean of two samples. # significant difference when compared juvenile and adults groups in females of *E. pusillus* ($p<0,05$).

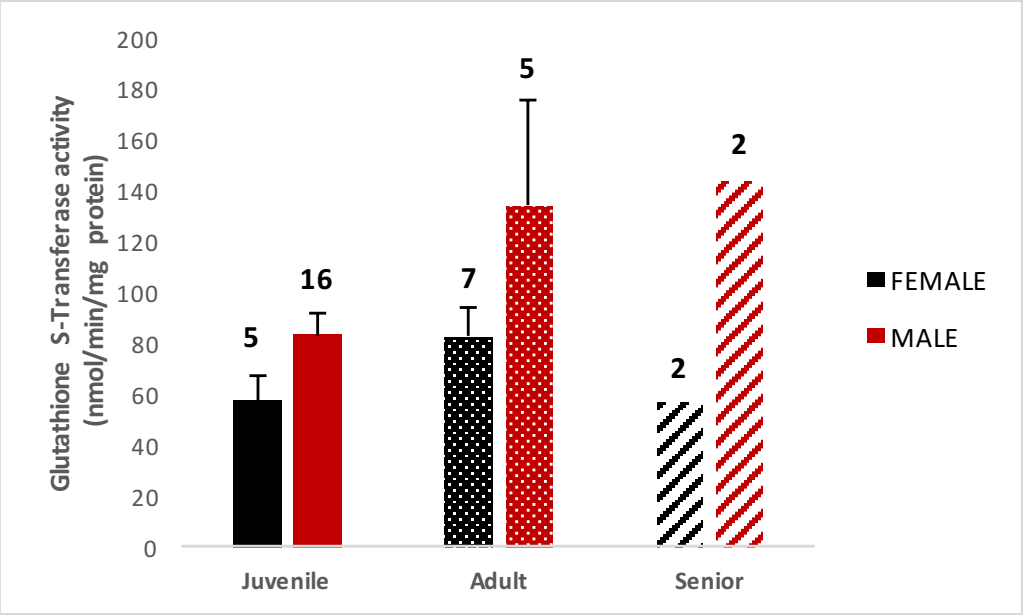

Figure 10-Comparative analysis of glutathione S-Transferase activity of liver tissues in female (N=14) and male (N=23) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). GST activity is expressed in nmol/min/mg of protein (mean ± SEM). Senior group was calculated by the mean of two samples.

Nonenzymatic antioxidants

Total Glutathione (TG) and Lipid peroxidation (LPO)

Glutathione total content in both genders (Figure 11) demonstrated a tendency do decrease across aging (lower levels in adult group). Juvenile males presented higher levels of TG when compared with juvenile females ($p<0.005$).

Levels of lipid peroxidation were significantly higher in juvenile males when compared by genders ($p<0.005$). Adult group presented significantly higher levels of LPO than juvenile group (Figure 12).

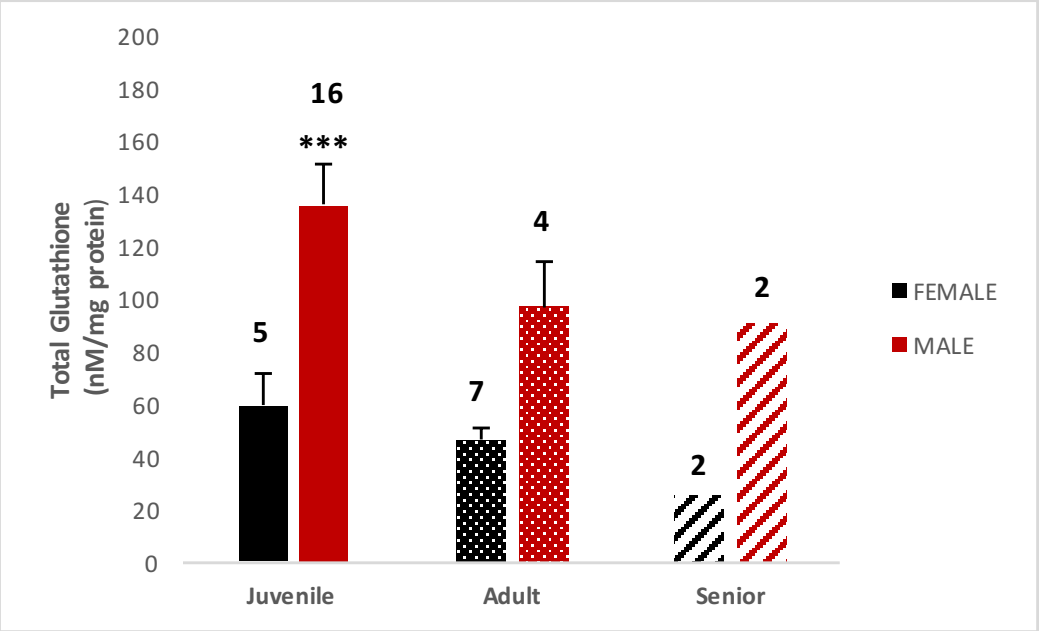

Figure 11-Comparative analysis of total glutathione (GSH/GSSH) of hepathic cells in male (N=22) and female (N=14) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). Total glutathione is expressed in nM/mg of protein (mean ± SEM). Senior group was calculated by the mean of two samples. *** significant difference between juvenile group of female and male (p<0,005).

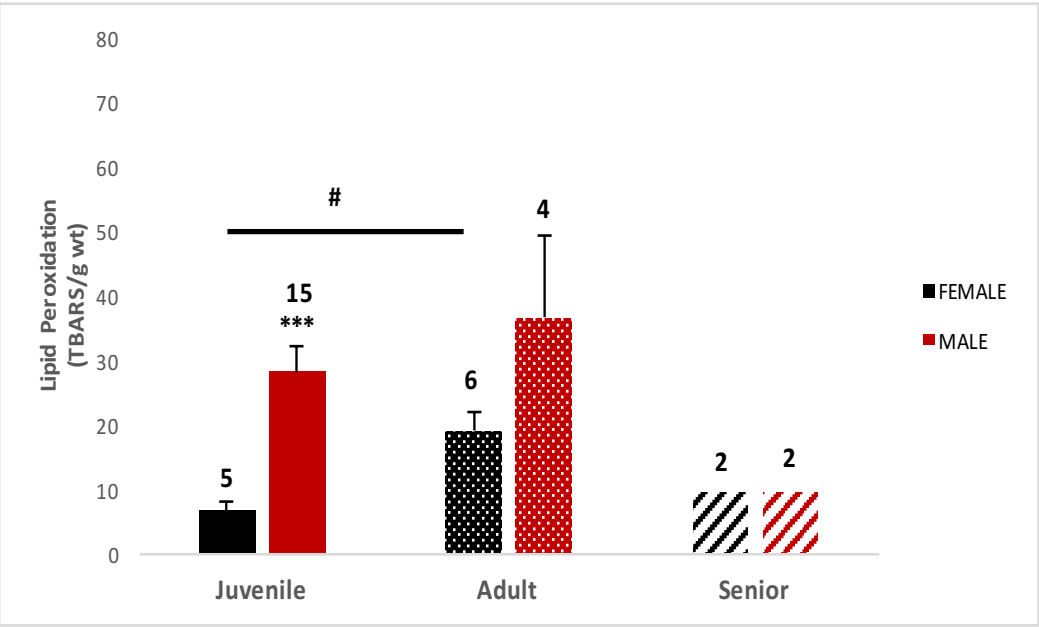

Figure 12-Comparative analysis of lipid peroxidation of liver tissues in male (N=21) and female (N=13) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). Lipid Peroxidation is expressed in nmol TBARS/g of wet tissue (mean ± SEM). Senior group was the result of the mean of two samples. # significant difference between juvenile and adult females in *E. pusillus* (p<0,05). *** significant difference between female and male in juvenile group (p<0,005)

Cellular energy allocation

Electron Transport System

At figure 13 are presented the results from the electron transport system for females and males of both species. Adult males presented significantly higher ETS activity than juvenile males ($p<0.05$). Adult females presented similar ETS activity than juvenile female ($p>0.05$).

Energetic Reserves

The quantification of the lipids content on hepathic tissues (Figure 14) showed significantly differences when compared juveniles from both genders ($p<0.005$) and a decrease across aging in male. Female of *E. pusillus* increase lipids content between juvenile and adult age but without significant differences (Figure 14).

Overall, the quantification of sugar content on hepathic tissue shows that this parameter of both genders in *E. pusillus* decrease along aging and are similar (Figure 15).

Figure 16 show the differences between protein content in both genders of *E. pusillus*. Meanwhile female have a protein content stable across aging, male have a decrease in adult group ($p<0.005$)

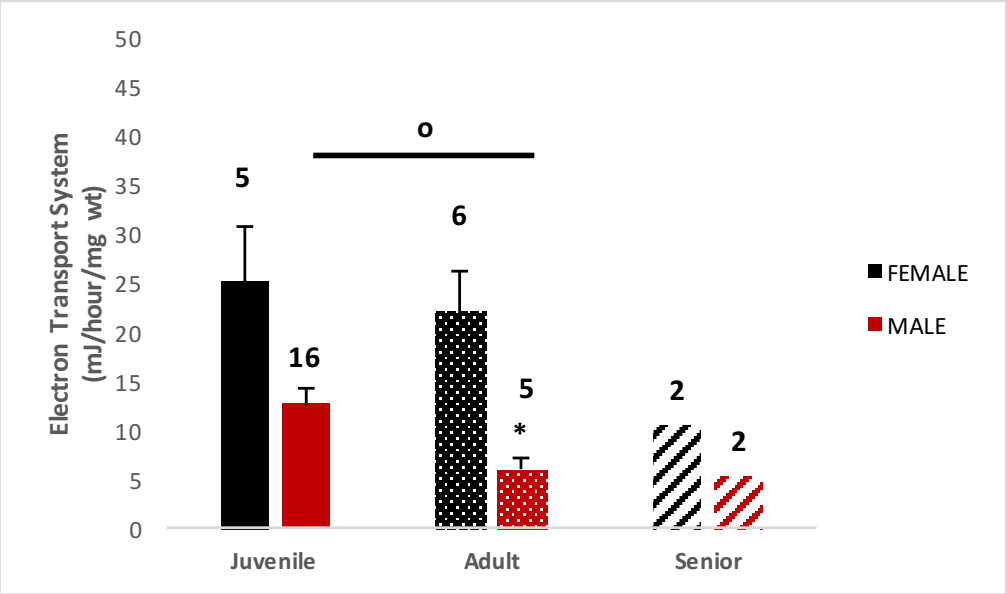

Figure 13 - Comparative analysis of electron transport system on hepathic cells in male (N=23) and female (N=13) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). Electron transport system is expressed in mJ/hour/mg of wet tissue (mean ± SEM).

Senior group was the result of the mean of two samples. ° significant difference between juvenile and adult males liver tissues in *E. pusillus* (p< 0.05). * significant difference between female and male adult´s (p<0,05).

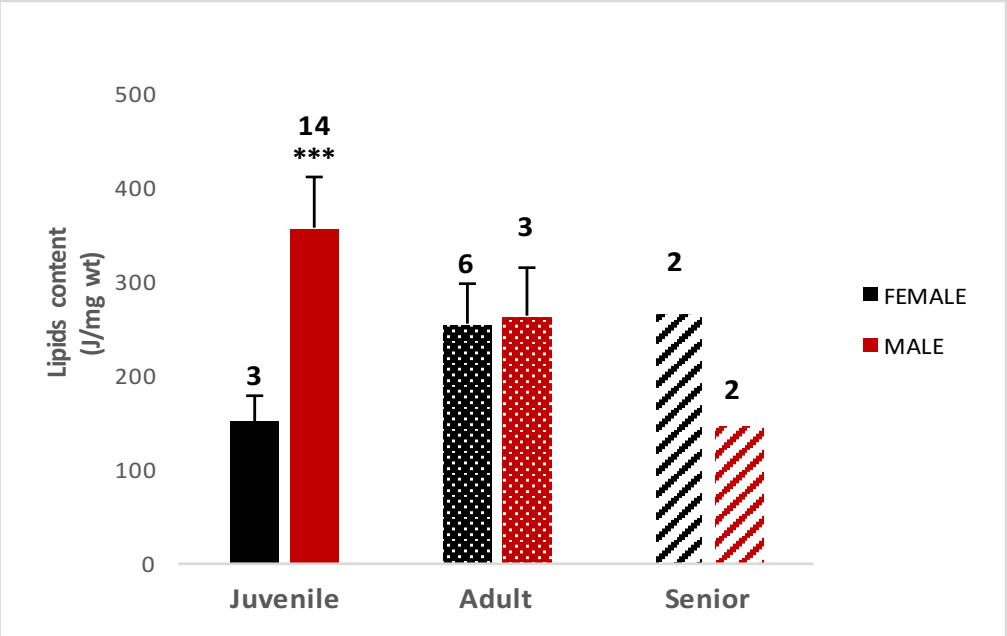

Figure 14-Comparative analysis of lipids content on hepathic cells in male (N=19) and female (N=11) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior). Lipids content is expressed in J/mg of wet tissue (mean ± SEM). Senior group was the result of the mean of two samples. *** significant difference between female and male juvenile´s (p<0.005).

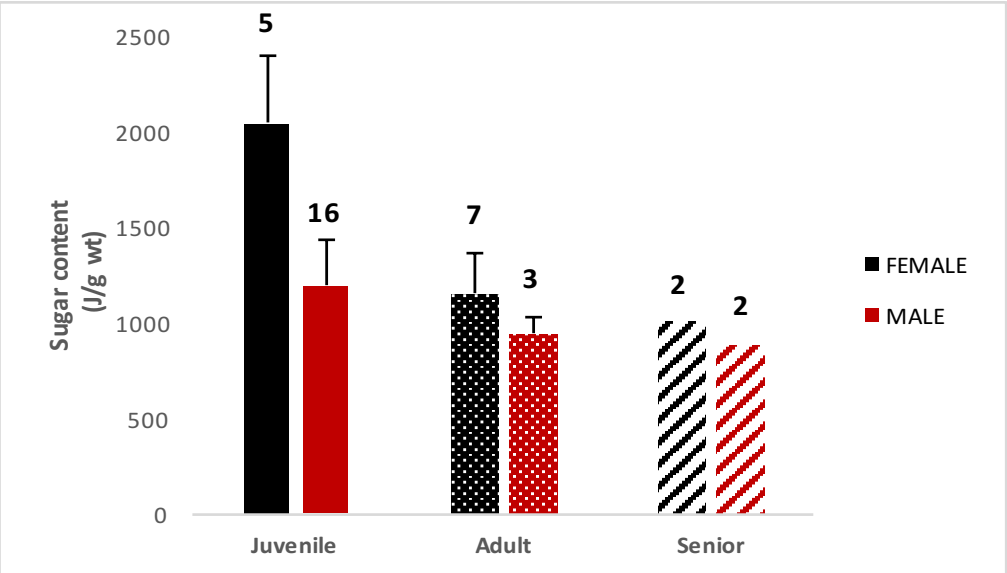

Figure 15-Comparative analysis of sugars content of hepathic cells in male (N=21) and female (N=14) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior).

Sugars content is expressed in J/g of wet tissue (mean ± SEM). Senior group was the result of the mean of two samples.

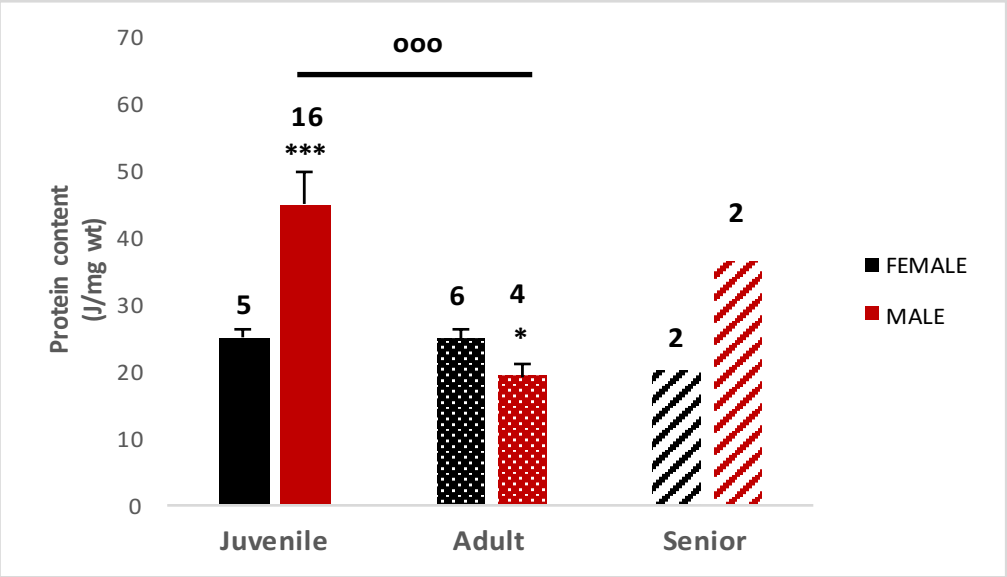

Figure 16-Comparative analysis of protein content oh hepathic cells in male (N=22) and female (N=13) individuals of *Etmopterus pusillus* at three different ages (juvenile, adult and senior) with bovine serum albumin as standard. Protein content is expressed in J/mg of wet tissue (mean ± SEM). Senior group was the result of the mean of two samples. * significant difference between female and male adults ($p<0.05$). *** significant difference between female and male juveniles ($p<0.005$). ^{ooo} significant difference between juvenile and male males ($p<0.005$).

Galeus melastomus

Enzymatic Antioxidants defences

Catalase (CAT) and Glutathione S-Transferase (GST)

In the different age groups of *G. melastomus,* the levels for catalase activity keep stable: juvenile (≈ 58.45 µmol/min/mg protein), adult (≈ 58.27 µmol/min/mg protein) and senior (≈ 57.51 µmol/min/mg protein). Adult females presented similar catalase activity than juvenile female (Figure 17).

Significant differences are shown for GST activity in hepathic samples of G*. melastomus* between juvenile and adult groups ($p<0.05$) (Figure 18).

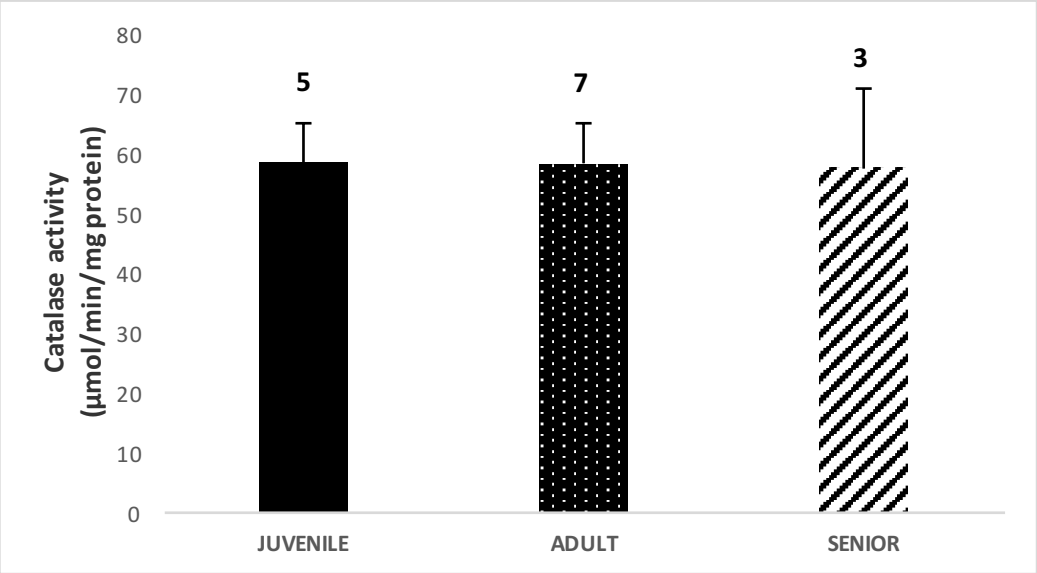

Figure 17- Catalase activity at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus (N=15)*. Catalase activity is expressed in µmol/min/mg of protein (mean ± SEM).

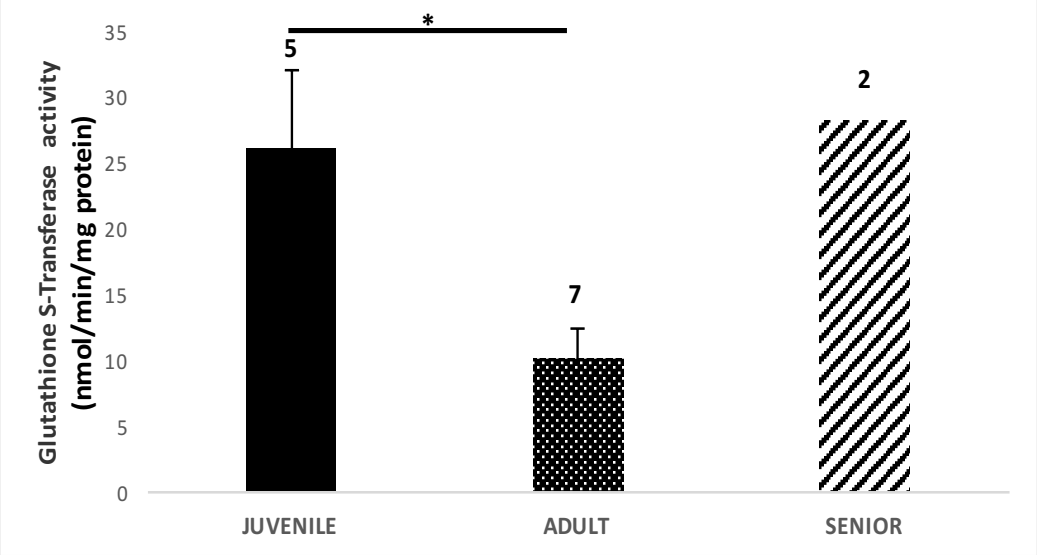

Figure 18-Glutathione S-Transferase activity at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=14). Glutathione S-Transferase activity is expressed in nmol/min/mg of protein (mean ± SEM). Senior group was the result of the mean of two samples. * significant difference between juvenile and adult groups in *G. melastomus* ($p<0,05$).

Nonenzymatic antioxidants

Total Glutathione (TG) and Lipid Peroxidation content (LPO)

G. melastomus hepathic tissues show a slightly increase of total glutathione across aging: juvenile (≈ 26.30 nM/mg protein), adult (≈ 34.07 nM/mg) and senior (≈ 53.78 nM/mg protein). Adult females presented similar glutathione levels than juvenile female (Figure 19).

Levels for lipid peroxidation in hepathic tissues of *G. melastomus* are higher in juvenile group and when compared with adult ($p<0.05$) and senior($p<0.05$) the differences are significant (Figure 20).

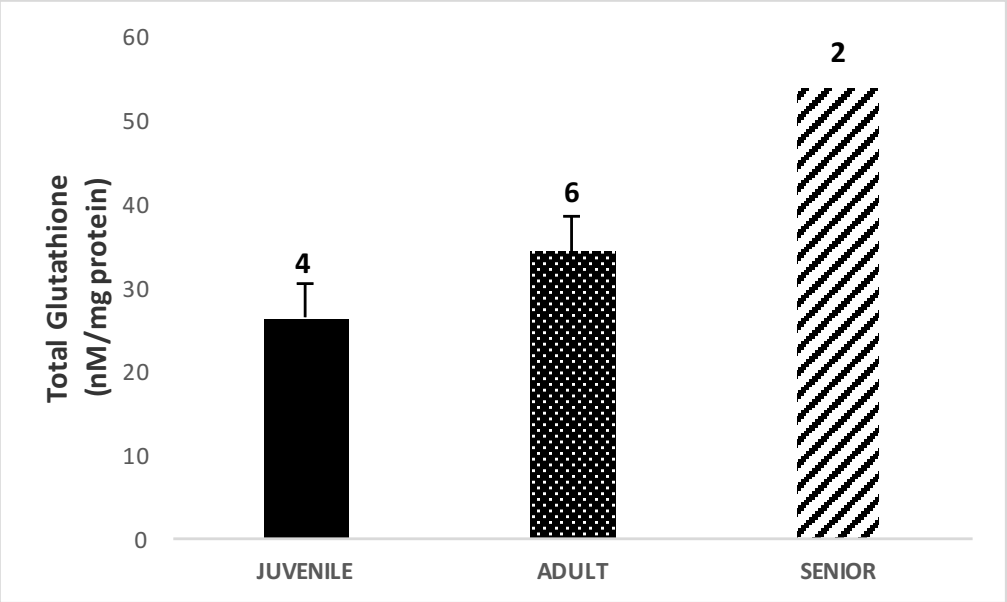

Figure 19-Total glutathione at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=12). Total glutathione is expressed in nM/mg of protein (mean ± SEM). Senior group was the result of the mean of two samples.

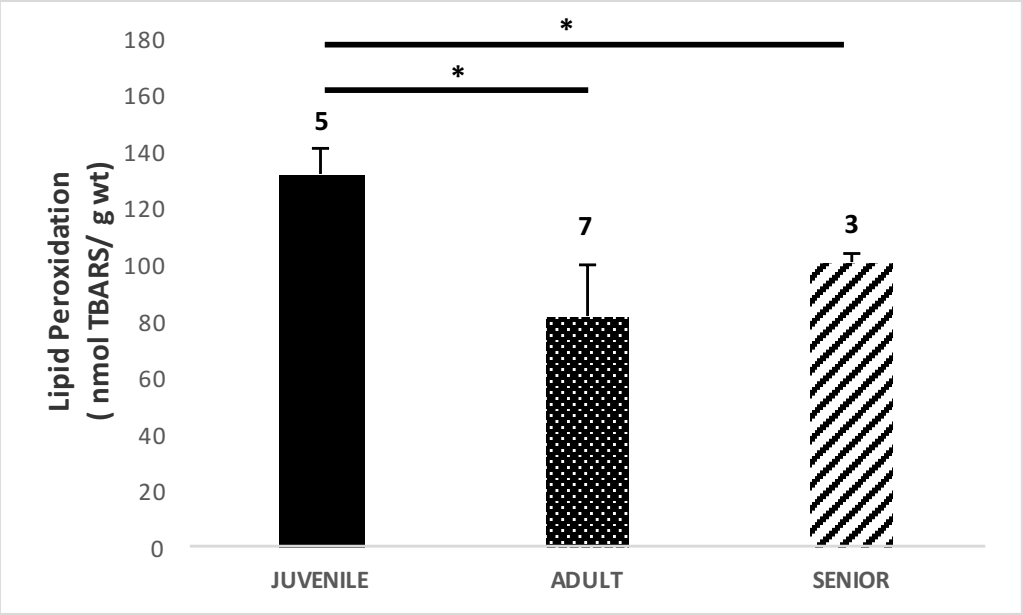

Figure 20-Lipid peroxidation at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=15). Lipid peroxidation is expressed in nmol TBARS/g wet tissue (mean ± SEM). * significant differences when compared juvenile and adult and juvenile and senior groups of *G. melastomus* ($p<0.05$).

Cellular energy allocation

Electron Transport System

G. melastomus hepathic tissues show a slightly decrease across aging in the electron transport system and adult females presented similar ETS activity than juvenile female (Figure 21).

Energetic Reserves

In hepathic tissues of *G. melastomus* were observed higher levels of lipids contents at juvenile and adult group and a decrease in senior group (Figure 21).

Figure 22 show sugar contents in females of *G. melastomus*. Adult females presented similar ETS activity than juvenile female (Figure 22).

The concentration of reserve proteins decrease with aging: juvenile has the higher content and senior the lower, and when compared adult and senior group the difference is significant ($p<0.05$) (Figure 23).

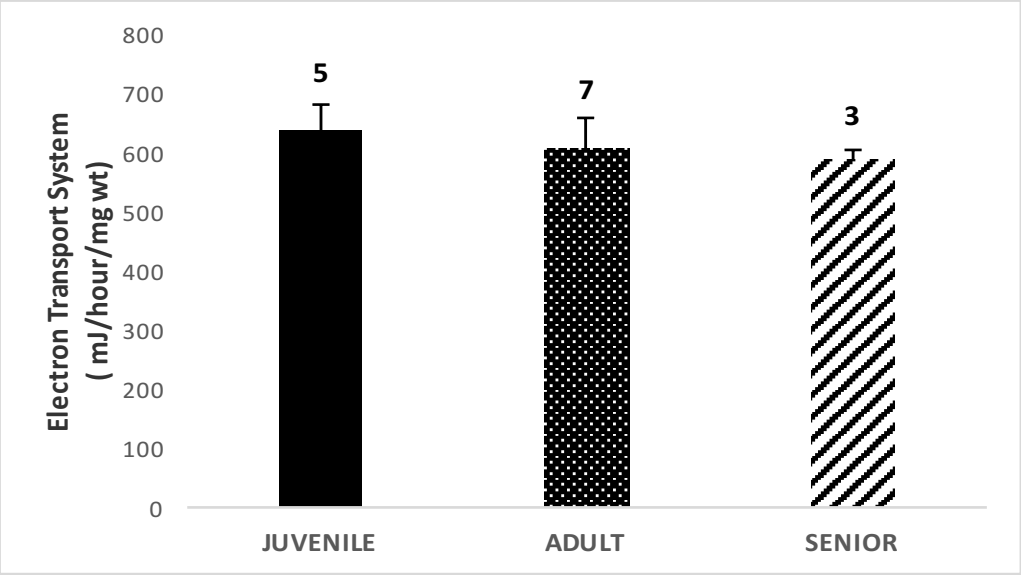

Figure 21 - Electron Transport system at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=15). Electron transport system is expressed in mJ/hour/mg of wet tissue (mean ± SEM).

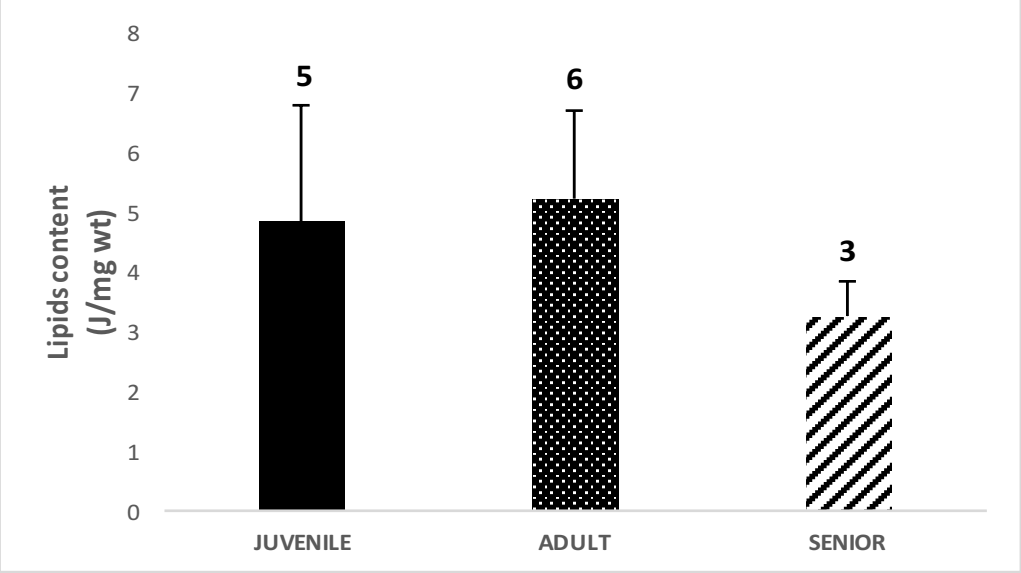

Figure 22 - Lipids content at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=14). Lipids content is expressed in J/mg wet tissue (mean ± SEM).

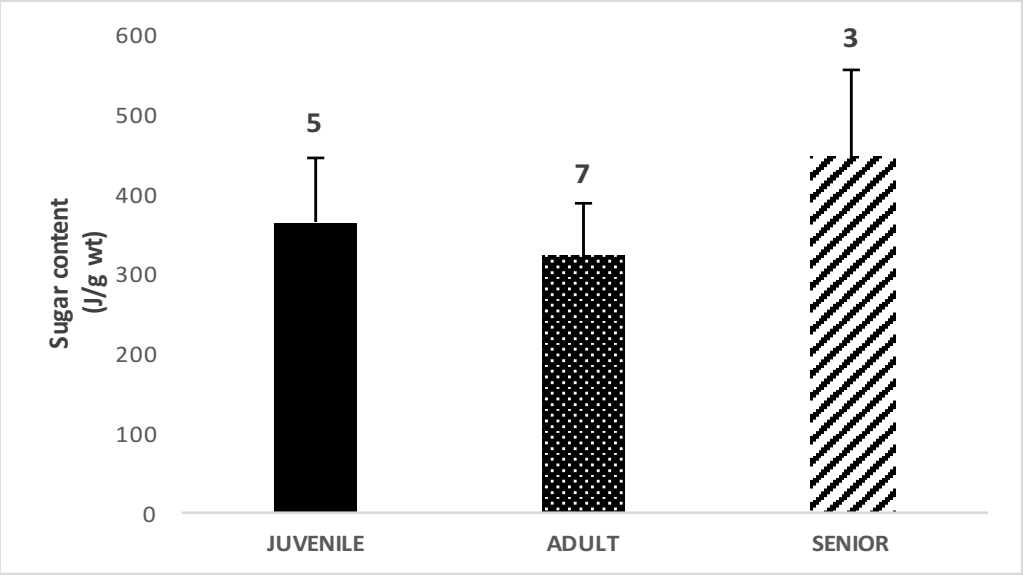

Figure 23 - Sugar content at three different age groups (juvenile, adult and senior) in hepathic cells of *Galeus melastomus* (N=15). Sugar content is expressed in J/g wt (mean ± SEM).

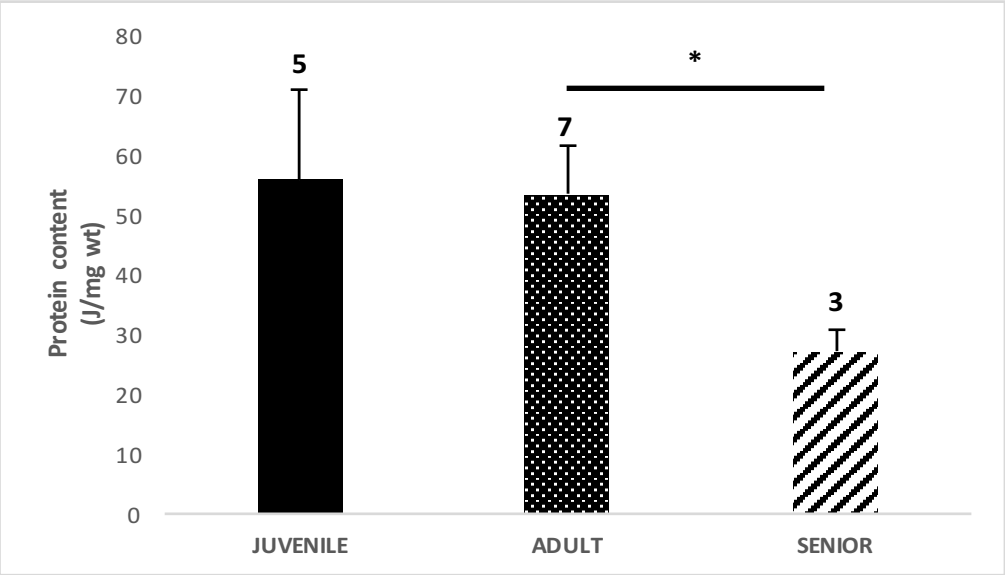

Figure 24 - Analysis of protein content female (N=15) individuals of *Galeus melastomus* at three different ages (juvenile, adult and senior) with bovine serum albumin as standard. Protein content is expressed in J/mg of wet tissue (mean ± SEM). * significant difference between adult and senior groups in *G. melastomus* ($p<0,05$).

IV-DISCUSSION

Elasmobranchs are an ancient group and is important to understand their adaptations and cellular processes which can influence the form as they face new levels of contaminants in their habitats.

In this discussion will be compared several biological parameters and the levels of PAH's in hepathic tissues of two shark species, *E. pusillus and G. melastomus*..

The results showed the presence of diverse PAH´s like naphthalene, phenanthrene, pyrene and benzo[a]pyrene in elasmobranchs liver tissue of both species. *G. melastomus* presented higher levels of PAHs in liver tissue than *E. pusillus*. Since both species are deep-water sharks, this result could suggest a better and efficient detoxification system in *E. pusillus* in relation to organic PAHs in liver tissues. Several authors conclude that demersal or benthic species have an bigger uptake of contaminants (Gravato *et al*., 2014; Mountouris *et al*., 2002; Rudneva, 1999). Overall, the appearance of organic xenobiotics with carcinogenic and mutagenic properties in tissues of aquatic animals is a warning for toxic state of marine environments.

To protect from xenobiotics exposure, organisms have antioxidants defences to maintain oxidative stress in levels that can be supported by the organism.

Catalase activity dissociate hydrogen peroxide (a ROS specie) into water and oxygen gas (Chelikani *et al*., 2004). Normally, catalase activity increase across aging as a result of increasing levels of non-scavenged ROS *(*Martínez-Álvarez *et al.*, 2005). This is according with results found in present study, as *E. pusillus* adult males presented higher CAT activity than juvenile males. However, in the case of *G. melastomus*, catalase activity don´t show any significant differences by keeping stable across aging. Montserrat Solé (2010) report that *G. melastomus* have some enzymatic and non-enzymatic metabolism differences when compared with other elasmobranchs.

Glutathione S-transferase activity, which is responsible for phase II of detoxification and a marker of antioxidant mechanisms, showed higher activity in adult group of *E. pusillus* that might be related to higher levels of PAHs, principally

naphthalene (PAH detected in higher levels in hepathic samples). Gravato and Guilhermino (2009) concluded that GST activity can increase until a certain PAHs concentration but decrease when organisms are exposed to higher concentrations. This could be explained by partial inhibition of the pathway for glutathione conjugation in detoxification process and by other defences which can be activated to equilibrate the state of oxidative stress.

Redox pair GSH/GSSG it´s one of the most important antioxidant defence and have a critical role in stabilize the levels of H_2O_2. For both genders of *E. pusillus* results showed a decrease in TG across age groups until reach the lower values at adult group this could be explained by the ability of glutathione to fight oxidative damage induced by pollutants. However, more studies are needed to better understand this metabolic pathway in sharks.

Lipid peroxidation is a biomarker that shows the effect of ROS in cellular membrane by the lipids damage and could provoke inactivation of proteins and DNA adducts formation (Montserrat Solé *et al*., 2010). Lipid peroxidation on both genders of *E. pusillus* increased in both genders of adults. Peroxidation of lipids is related to damage caused by ROS on biological lipids (M. Solé *et al*., 2008). Being ROS levels the main cause for lipid peroxidation, results from this study could indicate that PAHs levels present in liver tissues might be directly related with the membrane damage and lipids oxidation. *G. melastomus* lipid peroxidation levels in liver samples denote a decrease in adult and senior groups that might be justified by the higher GST activity found in these ages, as showed by Üner *et al*. (2001). for other fish species.

Rudeneva (1999) report the importance of the knowledge of antioxidants defences in early stages of embryogenesis development of studied organism to understand the defences mechanisms on life cycle. Rueda-Jasso (2014) conclude that a diet rich in row carbohydrate induce oxidation, quantify enzymatic and non-enzymatic antioxidant defences, e.g. SOD and EROD. Ecological parameters will be important to a better understanding, like feed behaviour, stomachs content and reproductive cycle.

Relatively to oxygen consumption in hepathic tissues, results showed a slightly decrease across aging in both genders fact that might be explained by the high rates of swimming activity in sharks (Martínez-Álvarez *et al.*, 2005). Oxygen

consumption in *G. melastomus* remains stable across life cycle but when compared both species results *G. melastomus* showed significantly higher metabolic rates (Figure 25).

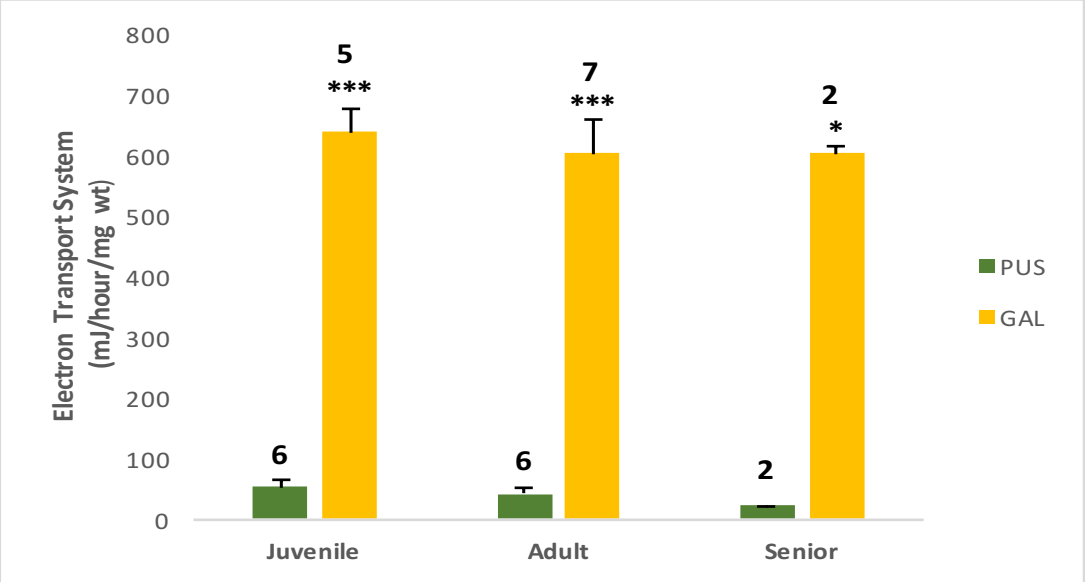

Figure 25- Comparative analysis of oxygen consumption between *Etmopterus pusillus* and *Galeus melastomus* females in hepathic tissue at three different ages (juvenile, adult and senior). Electron transport system is expressed in mJ/hour/mg of wet tissue (mean± SEM). * significant difference between *E. pusillus* and *G. melastomus* females seniors ($p<0.05$) *** significant difference between *E. pusillus* and *G. melastomus* females ($p<0.005$).

These results could be explained by differences on feeding habits which could imply higher rates of swimming for looking preys at 1600 meters depth (Anastasopoulou *et al., 2013)*.

Lipids and protein contents in liver are directly influenced by accumulation of non-scavenging ROS and membrane damage with consequences at level of proteins and lipids degradation (Martínez-Álvarez *et al*., 2005). In the case of *E. pusillus* male and *G. melastomus* female individuals, results showed a decrease in lipid content across life cycle that could indicate a large exposure to PAHs and consequently lipid degradation. On the other side, female individuals showed higher levels of lipids content in maturity age (adult) which can result from the reproductive condition or can resulted also from some different feeding behaviour dependent on season and bioavailability of preys (Fanelli *et al*., 2009; Anastasopoulou *et al*., 2013).

Due to its low commercial interest, to our knowledge, few studies on ecology, physiological and chemical metabolites of *E. pusillus* were performed. In this way, with this study we characterize a very small population of sharks in Atlantic Ocean in relation to several parameters, such as environmental exposure to PAHs and several biochemical processes related with oxidative stress and energetic metabolism.

Overall, the difficulty of controlling environmental (pH, salinity, different pollutants) and ecological (reproductive age, illness) factors is one of the main problems of field studies.

V-CONCLUSION

Relatively to homologous PAH´s quantification, the most common found in both species was naphthalene, and females of *Galeus melastomus* has higher levels when compared with *Etmopeterus pusillus*.

With the present thesis could conclude that enzymatic defence, catalase, increase his activity in adult stage thanks to long term exposure to homologous PAH´s. GST activity increase in adult group which can be related to lower PAH´s levels registered. Organism lipid levels decrease in adult group because exposure to PAH´s might damage and degrade membrane lipids.

It is increasingly clear that marine ecosystems are in real danger because humans treat it like garbage deposit. Each day, higher concentrations of PAH´s are present on the marine ecosystem and permanently in contact with aquatic organisms. Through gills and skin fish absorb high doses of organic xenobiotics that provoke, in a short period of time, sub-cellular damages at individual level and in habitat (pollutants became present in the food web).

Etmopterus pusillus and *Galeus melastomus* were the characterized species in this study and the principal goal was evaluated the exposure of both shark species to PAHs and the oxidative stress response at sub-cellular level. Independently of their low commercial value, it is extremely important to evaluate these shark populations, since they are a powerful tool to understand the consequences of pollution in water ecosystems.

By other side, the results showed that *Galeus melastomus* is an elasmobranch with some different characteristics, for example, a lipid different composition and higher levels of ETS. However, more studies should be made to understand the ecology, physiology and cellular mechanisms for this species.

In general, to complete associations with biomarkers results could make some more embraced studies that incorporate data like: I. Salinity, pH, temperature of the water, localization of specimen capture, II. Analyse stomachs contents, III. Understand the genetic mechanisms that command this metabolic pathway, IV. Understand the life cycle for this specie, V. Make a bigger analysis of diverse

contaminants concentration and understand in which way the metabolism is changed.

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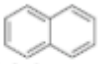
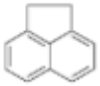
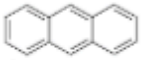
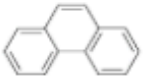
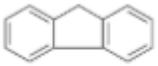
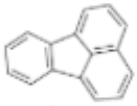
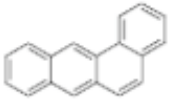
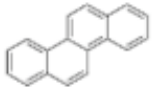
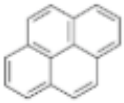
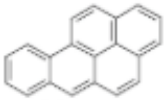
VII-APPENDIX

Table I - Chemical and Physical properties of 16 PAHs considered priority pollutants. Doyle et al, 2008.

PAH	Chemical formula	Molecular weight	Boiling point (°C)	Melting point (°C)	Solubility (mg l^{-1})	Physical appearance
 Naphthalene	$C_{10}H_8$	128	218	77	31	White crystals
 Acenaphthene	$C_{12}H_{10}$	154	279	95	3.8	White or pale yellow crystalline powder
 Acenaphthylene	$C_{12}H_8$	152	280	94	16.1	Yellow crystalline powder
 Anthracene	$C_{14}H_{10}$	178	340	218	0.045	Off-white to pale green crystals
 Phenanthrene	$C_{14}H_{10}$	178	340	99.5	1.1	White crystals
 Fluorene	$C_{13}H_{10}$	166	295	116	1.9	White crystals
 Fluoranthene	$C_{16}H_{10}$	203	375	111	0.26	Light yellow needles
 Benzo[*a*]anthracene	$C_{18}H_{12}$	228	438	158	0.011	Colorless to yellow-brown flakes
 Chrysene	$C_{18}H_{12}$	228	448	253	0.0015	Crystalline powder
 Pyrene	$C_{16}H_{10}$	202	404	145	0.132	Colorless to light yellow solid
 Benzo[*a*]pyrene	$C_{20}H_{12}$	252	495	179	0.0038	Yellow crystals

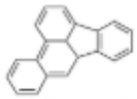
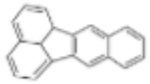
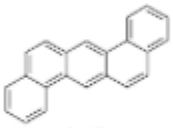
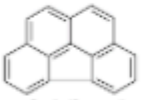
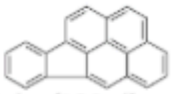
 Benzo[*b*]fluoranthene	$C_{20}H_{12}$	252	481	168	0.0015	Colorless crystals
 Benzo[*k*]fluoranthene	$C_{20}H_{12}$	252	480	216	0.0008	Yellow crystals
 Dibenzo[*a*,*h*] anthracene	$C_{22}H_{14}$	278	524	266	0.0005	White to yellow crystalline solid
 Benzo[*g*,*h*,*i*]perylene	$C_{22}H_{12}$	276	550	277	0.00026	Large pale yellow–green crystals
 Indeno[1,2,3-cd]pyrene	$C_{22}H_{12}$	276	536	162	0.062	Crystalline solid

Table II- *Etmopterus pusillus* list of individuals and morphometric parameters: total size(cm) and total weight (g) and gender (Mala-M and female – F).

SampleID	Haul	Date	Code	Taxa	TW_g	TL_cm	Sex
40	9	16072016	PUS	Etmopterus pusillus	361,55	50	F
43	9	16072016	PUS	Etmopterus pusillus	338	43,2	F
25	5	15072016	PUS	Etmopterus pusillus	288,4	42,1	M
53	9	16072016	PUS	Etmopterus pusillus	92,49	39,8	M
41	9	16072016	PUS	Etmopterus pusillus	277,5	39,5	M
32	7	16072016	PUS	Etmopterus pusillus	174,16	36,1	M
49	9	16072016	PUS	Etmopterus pusillus	213,45	36,1	M
22	5	15072016	PUS	Etmopterus pusillus	194,26	35,9	M
55	9	16072016	PUS	Etmopterus pusillus	184,39	35,6	F
34	7	16072016	PUS	Etmopterus pusillus	136,69	34,7	M
24	5	15072016	PUS	Etmopterus pusillus	157,89	34,1	F
52	9	16072016	PUS	Etmopterus pusillus	139,8	34	M
54	9	16072016	PUS	Etmopterus pusillus	146,53	33,2	F
44	9	16072016	PUS	Etmopterus pusillus	125,84	33,1	F
42	9	16072016	PUS	Etmopterus pusillus	138,08	33	F
56	9	16072016	PUS	Etmopterus pusillus	112,85	32,5	F
23	5	15072016	PUS	Etmopterus pusillus	118,34	30,6	F
50	9	16072016	PUS	Etmopterus pusillus	94,55	29,9	M
38	7	16072016	PUS	Etmopterus pusillus	91,73	29,7	M
33	7	16072016	PUS	Etmopterus pusillus	86,39	29,4	M
35	7	16072016	PUS	Etmopterus pusillus	77,55	28,1	M
37	7	16072016	PUS	Etmopterus pusillus	63,71	27,8	M
51	9	16072016	PUS	Etmopterus pusillus	73,31	27,8	M
57	9	16072016	PUS	Etmopterus pusillus	65,71	27,5	F
26	5	15072016	PUS	Etmopterus pusillus	72,53	27,3	M
39	7	16072016	PUS	Etmopterus pusillus	77,27	26,9	M
27	5	15072016	PUS	Etmopterus pusillus	64,86	26,1	F
36	7	16072016	PUS	Etmopterus pusillus	53,08	25,6	M
61	9	16072016	PUS	Etmopterus pusillus	54,09	25,5	F
60	9	16072016	PUS	Etmopterus pusillus	53,71	25,4	M
28	5	15072016	PUS	Etmopterus pusillus	52,79	25	M
58	9	16072016	PUS	Etmopterus pusillus	56,39	24,9	M
59	9	16072016	PUS	Etmopterus pusillus	40,34	23,4	F
63	9	16072016	PUS	Etmopterus pusillus	10,44	15,2	F
64	9	16072016	PUS	Etmopterus pusillus	9,63	14,6	M
65	9	16072016	PUS	Etmopterus pusillus	9,64	14,5	M
62	9	16072016	PUS	Etmopterus pusillus	9,19	14	M
66	9	16072016	PUS	Etmopterus pusillus	5,26	11,7	F

*T*able III- *Galeus melastomus* list of individuals and morphometric parameters: total size(cm) and total weight (g) and gender (Female – F).

SampleID	Haul	Date	Code	Taxa	TW_g	TL_cm	Sex
2	12	17072016	GAL	Galeus melastomus	203,19	41,3	F
3	12	17072016	GAL	Galeus melastomus	239,45	46	F
4	12	17072016	GAL	Galeus melastomus	166,8	39,9	F
5	12	17072016	GAL	Galeus melastomus	153,59	39,9	F
7	12	17072016	GAL	Galeus melastomus	182,35	40,5	F
8	12	17072016	GAL	Galeus melastomus	111,15	34	F
9	12	17072016	GAL	Galeus melastomus	55,65	26	F
11	12	17072016	GAL	Galeus melastomus	38,36	22,5	F
12	12	17072016	GAL	Galeus melastomus	37,71	22	F
13	12	17072016	GAL	Galeus melastomus	32,06	22	F
14	5	15072016	GAL	Galeus melastomus	48,9	25,9	F
15	5	15072016	GAL	Galeus melastomus	730,3	60,4	F
16	5	15072016	GAL	Galeus melastomus	576,92	58	F
67	9	16072016	GAL	Galeus melastomus	195,78	40,2	F
76	9	16072016	GAL	Galeus melastomus	527,72	55,9	F