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Biologia marina

Valutazione quantitativa e macromolecolare di melanomacrofagi e lipidi epatici come strumenti promettenti per la conservazione degli elasmobranchi nel mare Adriatico

Quantitative and macromolecular assessment of hepatic melanomacrophages and lipids as promising tools for the conservation of elasmobranchs in the Adriatic Sea

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To my family,

for the support they gave me

throughout this journey

I say thank you.

Riassunto

Lo scopo di questo progetto consiste nell'andare ad identificare nuovi biomarker per la valutazione dello stato di salute degli squali pescati in Adriatico e la loro suscettibilità ai cambiamenti ambientali.

Per raggiungere questo obiettivo si è andati ad utilizzare i melanomacrofagi come biomarker di stress, già ampiamente utilizzati in teleostei, anfibi e rettili, in questi organismi.

In questo studio sono stati valutati i melanomacrofagi epatici in quattro specie di squalo pescate in Adriatico, sia da un punto di vista morfologico che quantitativo, valutandone le differenze fra le specie, fra i sessi e, dove possibile, fra questi ultimi ed il periodo riproduttivo. Infine, si è valutato se fosse presente una correlazione significativa tra i melanomacrofagi e l'età nei maschi di palombo.

Summary

Chapter 1

INTRODUCTION

1.1 Systematics

Chondrichthyes are a class of Vertebrates whose earliest fossils date back to the Devonian period (410-460 MA) of the Paleozoic Era. This fact shows the evolutionary success of this clade since it was able to overcome all five mass extinctions keeping its essential characteristics almost unchanged. The name of the class is formed from the Greek prefix *khondros*, meaning "cartilage" and suffiss *ikhthus* for "fish", due to the fact that their entire skeleton is made of cartilage.

This class includes 1223 species divided into 2 subclasses: Holocephali, which is made up of 52 species of organisms known as chimeras which all fall into the sole order of the Chimaeriformes, and Elasmobranchii (from the Greek, *elasmòs* = thin plate + from Latin, *branchia*), which are characterized by the presence of thin flaps of connective tissue that support the gills and separate the multiple gill openings vertically. This latter subclass is further separated into two subdivisions: Selachii, the sharks (532 species), and Batoidea, the rays (634 species) (IUCN, 2020).

Sharks are divided into 8 orders: Hexanchiformes, Squaliformes, Squatiniformes, Pristiophoriformes, Heterodontiformes, Lamniformes, Orectolobiformes and Carchariniformes. The first four are grouped into the superorder Squalomorphii. The species belonging to this clade have large spiracles while fin spines and anal fin can be present or absent and they have from 5 to 7 gill openings (Ari et al., 2008).

The last four orders are grouped into the superorder Galeomorphii. These species have five gill slits and an anal fin, lack fin spines and are presently the dominant group of sharks, found in all habitats in which sharks occur, except in the Arctic seas, which are inhabited by the squalomorph sleeper shark *Somniosus microcephalus* (http://species-identification.org/).

Most cartilagineous fishes have dermal denticles, better known as placoid scales, covering their skin to reduce drag. These anatomical structures are formed continuously during the life of the animal, they are shed and replaced as an individual grows. Shark' teeth are derived from dermal denticles which have become concentrated along the margin of the jaw and are organized in rows: the outermost row is continuously replaced by the next one.

1.2 General aspects

Sharks are found in all marine environments from the equator to the poles with a latitudinal gradient of biodiversity that decreases from the former to the latter. They occupy a wide range of habitats, from fresh and brackish waters to the open ocean and, as long as it's known, at all depths up to 3000m. (Ebert & Dando, 2021).

Sharks are carnivores with different feeding types ranging from filter-feeding to active predation. Most larger species are apex predators but there is a high diversity of mesopredators as shown by values of trophic level ranging from 3.1 (*Stegosoma fasciatum*) to 4.5 (*Carcharodon carcharias*), without considering the basking shark *Cetorhinus maximus,* the whale shark *Rhincodon typus* and the megamouth shark *Megachasma pelagios*, the three filter-feeding species of sharks (Cortés, 1999; Ferretti et al., 2010; Hobson & Welch, 1992). Features that allow these animals to be high in the food web are their hyostilic suspension of the jaw and their efficient dentition, which enable them to cut large prey into chunks, therefore consuming even larger animals. This is why many sharks are generalists in terms of prey items and why they play an important role in marine food webs, exerting a top-down control in ecosystem structure and functioning (Dulvy et al., 2014).

All Chondrichthyes have internal fertilization that occurs through a pair of claspers, intromittent organs derived from a modification of male pelvic fins. This mode of fertilization ensures that neither eggs nor sperms are broadcast haphazardly into the water column since there is a very high investment of energy of the mother in producing offspring to provide a direct development of the embryo.

Despite the unique mode of fertilization, Chondrichthyes display a wide range of reproductive strategies. About 43% of the species are oviparous (Compagno, 1990): in this case the embryos are encased in a hornlike egg case which is deposited in a safe place in the external environment. The rest of the species are viviparous and retain the embryos within the uterus throughout their entire period of development.

Viviparity can be divided into aplacental and placental viviparity depending on the trophic connection of the embryo with the mother.

Aplacental viviparity is a term that comprehends more than one mode of development:

- Yolk sac viviparity, better known as ovoviviparity, is the condition where the embryos gain nourishment solely from the yolk sac (lecithotrophic development) but they remain inside the mother's womb until they're able to be released into the external environment. Since there is no supplementary nourishment from the mother, youngsters are relatively small at birth.

- In aplacental oophagy uterine walls become large to accommodate a large number of eggs, mostly unfertilized, that will be the source of nourishment of one or few developing embryos which rely exclusively on their yolk sac until they develop a premature dentition that allows them to rupture the eggs and feed on their content. In the case of the sand tiger shark *Carcharias taurus* up to a dozen of embryos may be present in each of the two uteri and the fastest developing ones start to feed on their siblings, resulting in the parturition of two youngsters, one per uterus. This is known as intrauterine cannibalism and the result is a great size at birth, around 100cm, and an increased chance of survival.
- Dogfish sharks of the order Squaliformes and some sharks in the orders Orectolobiformes and Carchariniformes gain nourishment not only from their yolk sac but also from mucus secreted from cells on the surface of the uterus. This mode of nourishment is termed "histotrophy". In the stingrays of the order Myliobatiformes, many large projections with villi on them extend from the wall of the uterus toward the embryo, increasing the surface for the secretion of mucus

mostly composed of lipids, which have an high energy content. These projections are called trophonemata, and this kind of embryo's nourishment is named "lipid histotrophy".

In placental sharks, the yolk sac becomes highly vascularized and interdigitates with the uterine wall forming a placenta, through which nutrients are carried within the bloodstream from the mother to the embryo. The gestation period is unknown for most species but it has a wide range depending on the species and can reach up to 22 months in the spiny dogfish *Squalus acanthias*.

Depending on the species and on the body size of the mother, females may bear from 1-2 (as in the case of *Carcharias taurus*) to 300 youngs (whale shark *Rhincodon typus*).

Chondrichthyes exhibit a direct development, meaning that the youngsters are a miniature copy of the adult and they can survive into the external environment on their own, in fact parental care has never been documented. High natural survivorship is one of the general aspects that characterize this group together with slow growth, late age at maturity, long gestation periods, low fecundity and high longevity, all features that are typical of a K-selected life history strategy.

There is a general lack of knowledge for many aspects related to these animals and the information are mostly limited to charismatic species, commercially-important fisheries, and coral reef ecosystems (Dulvy et al., 2014). In addition there are logistic problems to the study of these animals such as data collection for highly migratory species or for species living in the deep-sea.

1.3 Threats

The rapid growth of the human population implies a higher demand for resources. To cope with the high demand for seafood, the fishing industry is increasing the pressure on the stocks. (Cataudella & Spagnolo, 2011; Dulvy et al., 2014; Ferretti et al., 2008) This industry faced a strong development after the Second World War thanks to the development of new technologies and the advent of commercial refrigeration which allowed to reach further distances for a longer period and store more products (Cavanagh et al., 2005). The result of this development was a stronger pressure on stocks, a decline of traditional food fish stock, and a consequent switch to lower-grade market fish.

Chondrichthyes are highly vulnerable to overfishing pressure because of their life history characteristics such as slow growth, low fecundity, and late maturity and it has been shown that populations of these animals can withstand only modest periods of fishing pressure which are followed by severe declines in catch rates, a cycle known as “boom-and-bust” (Cavanagh et al., 2005; Field et al., 2009). This is because, for longer-lived and less productive species, the initial rate of exploitation may exceed the rate at which the stock can replenish itself through reproduction, and right from the beginning the catches are unsustainable.

Many shark fisheries have collapsed in the past. One example is the one of the tope shark *Galeorhinus galeus* in the North-East Pacific: the fishery expanded greatly in 1936 for the high demand for shark’s liver’s oil, used as a source of vitamin A and for lubricant for military machinery before and during the Second World War, and then collapsed in 1944. The time span between boom and bust was eight years for this fishery (Klimley, 2013; Field et al., 2009).

Sharks are mainly fished as bycatch but commodities deriving from shark fishery are varied: they provide meat and fins for human consumption; leather; live specimen for aquaria; teeth, jaws, and branchial arches are sold as souvenirs; liver’s oil is used to produce lubricants, cosmetics, vitamin A, and it’s used also as an adjuvant in vaccines; shark’s cartilage is used in pharmaceutical for the treatment of arthritis and other ailments.

Particularly alarming is the market of shark fins which are a highly valued commodity in Asia, especially in China where there are records of this product as a delicacy since the Song dynasty (940-1279), and since then shark fin’s soup has been considered as a high-status meal (Cavanagh et al., 2005). According to FAO (Dent & Clarke, 2015) the trade of fins generates a profit of around 100 million dollars higher than the one of shark meat even though the quantity of product is around a seventh of the former in respect of the latter.

Since sharks are fished mainly as bycatch in fisheries targeting other species of higher value and since fins are significantly more valuable than meat, fishermen cut off the fins of sharks they catch and discard the rest of the body at sea, often still alive. This practice is known as “finning” and fins are stored together with target species since they don’t take up much storage space as the whole animal. This practice is illegal and unethical and there’s a tremendous lack of data because there is no information of the exact species traded, landings may often be unreported, and the market is globally widespread if considering both exporters and importers therefore there is a difficulty in tracking the source.

According to the IUCN Red List, the main threat is overfishing but others are habitat loss and pollution. Life histories of sharks are quite varied depending on the species and many still need to be understood but generally, sharks move from mating areas, to nursery areas, and to feeding areas, therefore, requiring more habitats: loss of coastal habitats, or other kinds, can therefore compromise the recruitment for some species.

Pollution is a threat to all kinds of environments and can affect Chondrichthyes both directly or indirectly, through changes in water quality and habitat degradation. Generally, these animals are highly mobile so they can move from a place of less quality to a better one but certain life stages confined to a limited environment are more at risk.

Chondrichthyes can also bio-accumulate heavy metals and other pollutants such as organic chemical compounds that can have implications on the reproductive, immune, endocrine, and nervous systems.

Another source of pollution is the disruption of natural electro-magnetic fields around undersea cables since these animals rely on their electrosensory system for foraging and for migrating (Field et al., 2009).

1.4 Sharks in the Mediterranean

The Mediterranean hosts 76 species of Chondrichthyes (2 Chimaeriformes, 31 Batoidea, and 43 Selachi), around 6% of the 1223 species known globally, enough to consider the basin a hotspot for these animals. However, a total of 49 species (64,4% of the total) are considered among the threatened categories of the IUCN Red List (Vulnerable,VU; Endangered, EN; Critically Endangered, CR) which makes them the most endangered group of fish of the basin. The Mediterranean sea is at the same time the area in the world with the highest proportion of threatened species because of unregulated fishing (Bradai et al., 2018). The distribution of these species is not homogenous throughout the basin (Serena, F. 2005) and some habitats are considered critical for this group, even though there is a big lack of knowledge about it (Bradai et al., 2018). The biodiversity of elasmobranchs is highest in the western coastal Mediterranean while decreases going toward the eastern Mediterranean.

Species found in the Mediterranean can be split into 3 ecological groupings based on where they live in relation to continental landmasses and seabed: continental shelf species live from the coastal shore to depths of 200m; deepsea species live close to the seafloor at depths below 200m; oceanic species have no relation with the seafloor and live primarily away from continental landmasses or close to offshore islands where the continental slope is not far from the coastline.

Some species are pelagic, others spend their whole life on the seabed and are considered benthic, others swim into the water column but feed mainly on benthic species and these are considered benthopelagic (Ebert & Dando, 2021; (Cataudella & Spagnolo, 2011). The majority (around 80%) of Mediterranean elasmobranch are coastal and benthic.

The Mediterranean has been inhabited for millennia and it still has a high density of inhabitants along its coasts and a high influx of tourists each year and which results in a high anthropic pressure especially along the coastal areas of the basin, where fishing efforts also are more concentrated.

FAO statistics (1980-2015) show that landings of elasmobranchs decreased over the years while CPUE (Catch Per Unit Effort) generally increased. Many works have pointed out this statement (Jukic-Peladic et al., 2001; (Ferretti et al., 2008); Cataudella & Spagnolo, 2011; Robbins, 2014) and shown that, at least since the first decades of the last century, demersal communities in the Mediterranean were more diversified thanks to the presence of large predators while now the communities are characterized by species with faster growth and higher fecundity.

Elasmobranchs are mainly caught as bycatch but there are also examples of target fishery in the basin: in Tunisia and Libya after the decrease in swordfish catch, longlines that were previously used for that fishery are now used to catch sandbar shark *Carcharhinus plumbeus*; in North Adriatic and in a restricted area of the Turkish mediterranean coast gillnets are used to target *Mustelus* spp., which is the most targeted species of elasmobranch in the whole Mediterranean due to its distribution in the basin.

Cartilagineous fishes are caught accidentally in most fishing gears in the Mediterranean both in small-scale and industrial fisheries:

- Surface longlines that target tuna and swordfish affect pelagic sharks such as blue shark *Prionace glauca* and shortfin mako *Isurus oxyrhynchus*;
- Bottom longlines that target groupers affect more benthic sharks such as the ones of the genus *Mustelus*;
- Trawlers affect all species of elasmobranchs, particularly demersal species such as *Mustelus* spp., *Scyliorhinus canicula,* and *Squalus acanthias*.

Elasmobranch situation has not passed undetected and since the last decade of the twentieth-century efforts are being made to mitigate pressure on these animals:

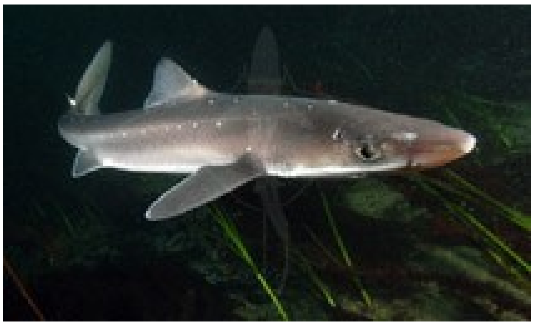
- 1996: European Elasmobranch Association (EEA) is established for coordinating the activities of national European organizations dedicated to the study, management, or conservation of chondrichthyans (sharks, skates, rays, and chimeras).
- 1999: FAO adopted the International Plan of Action (IPOA-Sharks) to ensure the conservation and management of sharks, skates, rays, and chimeras fished in territorial waters of the countries of the UN, and their long-term sustainable use.
- 2003: EU adopts the first finning regulation (No 605/2013) allowing sharks to be processed on board by removing their fins from their bodies and providing a "fin-to-carcass" ratio, to ensure that there is a correlation between the weight of a shark's fins and its body. Fins and bodies can be landed in different ports.
- 2004: ICCAT (Internation Commission for the Conservation of Atlantic Tunas) adopts shark finning regulation of the previous year.
- 2012: GFCM (General Fishery Commission for the Mediterranean) prohibits retention of 24 species of elasmobranchs listed in Annex II

(Endangered and threatened species) of the Barcelona Convention SPA/BD Protocol.

- 2013: EU adopts Fins Naturally Attached (FNA) for simpler and more effective monitoring and enforcement.
- 2018: GFCM adopts FNA
- 2019: ICCAT adopts the first international catch limit in the Atlantic for blue shark *P.glauca*.
- 2020: EU adopts blue shark quota; UNEP/MAP Cartilaginous Fishes Action Plan update.

1.5 Species used in the study

SQUALUS ACANTHIAS



Order: Squaliformes

Family: Squalidae

Ecology

This species has a worldwide distribution except for the tropical and polar regions and the north Pacific, where it's replaced by *S.suckleyi* (Bargione et al., 2019). It occupies a variery of habitats from estuaries to the upper continental slope but it is more common on the continental shelf in waters between 7 to 15°C. It is a highly migratory demersal shark but can be found also in midwater and at the surface, that moves in foraging schools up to thousands of individuals usually segregated by size and/or sex (www.fishbase.org).

Its diet is composed of bony fishes, cephalopods and crustaceans.

Reproduction:

It is an aplacental viviparous species with a very long gestation period from 18 to 24 months. In the Adriatic sea, pregnant females carrying nearly full-term embryos have been recorded in summer while fluent males were found all year round. The number of litters varies from 1 to 21. There is no resting period between one pregnancy and another, as soon after the parturition a female ovulates and is ready to conceive again.

Size at birth is constant through the Mediterranean basin and ranges between 245 to 271 mm (Bargione et al., 2019; Capapé & Reynaud, 2011); size at sexual maturity (L_{50}) in the Adriatic is 504mm for males and 725mm for females; age at maturity is between 9-11 years for males and 10-20 years for females; depending on the geographic area they live in, individuals living in colder waters take more time to grow and to reach sexual maturity and they are more longevous, they can live more than 40 years and the maximum recorded age is 75 years.

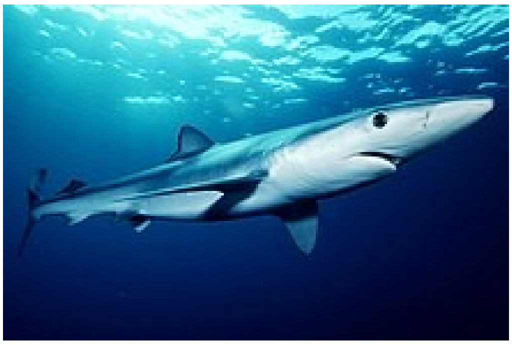
IUCN Status

Global: Vulnerable

Mediterranean: Endangered

Its distribution overlaps to that of the areas most subject to fishing effort especially in the northern-central Adriatic where the sea bed is entirely accessible to towing gears. Moreover its gregarious nature increases its captability. Females aggregations are found in deeper waters than male ones except for gravid females aggregations, which are found in more protected bays where they give birth.

PRIONACE GLAUCA



Order: Carchariniformes

Family: Carcharinidae

Ecology:

It is a highly migratory pelagic species found in all marine environments between 71°N and 55°S, occurring from the surface to at least 1160m, but mostly epipelagic. It is an oceanic species but it can be found close to the coast where the continental shelf is narrow and it has a water temperature range from 7°C to 21°C.

It feeds on bony fishes, cephalopods, smaller chondrichthyans, crustaceans and cetacean carrion.

Reproduction:

It is a placental viviparous species with a gestation period that ranges from 8 to 12 months depending on the environmental conditions of the habitat it lives in, but for the Mediterranean it has been calculated to be around 8 months

(Megalofonou et al., 2009). Parturition occurs between spring and summer and litters average 30 pups (up to 135 have been recorded).

Size at birth is calculated to be 30,6cm; size at sexual maturity (L_{50}) in the Mediterranean is 202,9cm for males and 214,7 cm for females (Megalofonou et al., 2009); age at maturity is 4-6 years for males and 5-7 years for females. According to Megalofonou et al., 2009, blue shark in the Mediterranean sea reaches sexual maturity in a smaller size but at the same age than the atlantic blue shark.

Mediterranean population is considered independent from the Atlantic population but the extent of exchange between population, if there is any, has not being understood.

IUCN Status

Global: NEAR THREATENED

Mediterranean: CRITICALLY ENDANGERED

This species is caught globally as target species or by-catch mainly by longlines, purse seine and gillnets both in small and industrial fisheries.

In the Mediterranean this species showed a dramatic decline in abundance and biomass of 96,5-99,8% from the beginning of 20th century to the beginning of the 21st (Ferretti et al., 2008).

It is one of the most traded species in the fin market contributing to 34,1-64,2% of the total traded (Fields et al., 2018).

MUSTELUS MUSTELUS

Order: Carchariniformes

Family: Triakidae

Ecology:

Its areal of distribution comprises the Eastern Atlantic from South Africa to the UK and the Mediterranean sea. It is a demersal species that lives on the continental shelf till the margin of the continental slope on sandy or muddy substrates and in a water temperature range from 10-21°C. It is commonly found between 5-50m but it’s been observed at 800m.

It feeds on mostly on crustaceans that represent the dominant fraction of the diet, bivalves, cephalopods and bony fishes.

Until recently *Mustelus mustelus* and *Mustelus punctulatus* were misidentified therefore the exact distribution of each species is not completely known, however historical data show a restriction in their distribution in the Mediterranean and now they are more commonly found in the Adriatic sea and the Strait of Sicily (Riginella et al., 2020).

Reproduction:

Smooth-hounds are viviparous placental species and both *M.mustelus* and *M.punctulatus* have a gestation period of 11 months with parturition occurring in April-May. All *Mustelus* species have a short resting period between pregnancies, around 4-6 weeks for specimen studied in the Gulf of Gabès, and large oocytes in post-partum females indicates that vitellogenesis proceeds also during pregnancy (Saïdi et al., 2008). Fecundity is related to female size in both species and is higher in *M.punctulatus* (mean fecundity = 24) rather than *M.mustelus* (mean fecundity = 11).

In the north Adriatic Sea, size at birth is 37cm for M.mustelus and 25cm for *M.punctulatus*; L_{50} is 108,1cm and 91,3cm for males and 121,2 cm and 109,9 cm for *M.mustelus* and *M.punctulatus* respectively, which are higher than the one found in the Gulf of Gabès and in the Gulf of Iskenderun showing a latitudinal gradient (Riginella et al., 2020); data on age at maturity in the

Mediterranean and longevity are available only for *M.mustelus* and are 8 and 25 years for females and 7 and 20 years for males (Ozcan & Başusta, 2018).

IUCN Status

	M.mustelus	M.punctulatus
Global	ENDANGERED	VULNERABLE
Mediterranean	VULNERABLE	VULNERABLE

Smooth-hounds in the Mediterranean are captured with demersal trawls, trammel nets, gillnets and longlines and landings dropped significantly after 1994 but species-specific data are not available since all smooth-hounds are considered the same species after landing (www.redlist.org). However, given the life history traits of these species, *M.mustelus* is more vulnerable to fishing than *M.punctulatus*.

SCYLIORHINUS CANICULA

Order: Carchariniformes

Family: Scyliorhinidae

Ecology:

Small species of shark distributed in the eastern Atlantic from Senegal to Norway, in the Mediterranean, and the Black Sea. It is a demersal species inhabiting the continental shelf and the uppermost slope from 10-800m depth. It is found on a variety of substrates, from muddy to sandy to gravelly and coralline bottoms.

It feeds on benthic invertebrates such as bivalves, crustaceans, small cephalopods, polychaetes e small bony fishes.

Since it doesn't have pelagic stages and it has a high site fidelity, deep marine areas act as a geographical barrier to its dispersal, in fact, in the Mediterranean, the small-spotted catshark has a strong genetic differentiation (Vasiliki Kousteni & Megalofonou, 2019).

Reproduction:

S.canicula is an oviparous species that produces one egg per oviduct and lays them simultaneously. The egg cases have tendrils used to secure their attachment on macroalgae, seagrass, or another solid substrate. Eggs hatch after 5-11 months depending on water temperature (Koehler et al., 2018).

The egg-laying period is carried out through the whole year with resting periods of few weeks or none, depending on the geographical area and females can store sperm for a long time in the oviductal gland (Christian Capapé et al., 2008; Finotto et al., 2015). The lack of a seasonal pattern of GSI in males suggests that they can mate throughout the year.

The size of egg cases depends on the geographical area and the size of the female and ranges from 4,9-7,0cm in length and 1,5-3,0cm in width; L50 for the Adriatic is estimated to be around 360cm and the maximum length is estimated to be around 490cm, both measurements are higher than the corresponding measures for small-spotted catsharks in the Strait of Sicily; females can lay from 40 to 240 eggs each year.

Females are slightly smaller than males and this size dimorphism is different from the majority of elasmobranch species: while in placental and aplacental viviparous species and oviparous species that lay eggs in a short time a greater size of the female corresponds to a greater fecundity, in an oviparous species like S.canicula that lays eggs throughout the year this selective pressure is not applied since fecundity is not related to female size (Christian Capapé et al., 2008).

IUCN Status

Global: LEAST CONCERN

Mediterranean: LEAST CONCERN

It is caught as bycatch from trawlers, longlines and gillnets but despite ongoing fishing pressures it is considered one of the most abundant elasmobranchs in the Mediterranean Sea.

1.6 Melanomacrophages

Biomarkers are any kind of measurable/evaluable biological indicator on any biological state and are divided into two categories: “exposure biomarkers” which are measurable quantitative alterations at a molecular, biochemical or cellular level, that provide precise indications on the class of contaminants involved; and “effect biomarker” that reveal the presence of an alteration but don’t provide any indication on the trigger. Some biomarkers have a prognostic value while others have a diagnostic significance and they are a measure of some effect that was, or still is, present.

Melanomacrophages (MMs) are considered an effect biomarker with a diagnostic value. These cells are present in most fish, amphibians, and some reptiles and they appear darkly pigmented due to the high content of melanin, lipofuscin and/or haemosiderin. They appear after the first feeding episode and are found in the liver, spleen, and head kidney (Agius, 1981).

Their primary activity is phagocytosis of endogenous (cells) or exogenous material (heavy metals, bacteria, pollutants) difficult to process and/or toxic, however, they also participate in immune defenses: MMs aggregate in centers (MMCs) and these are the place where the antigen, uptaken by MMs, can react with lymphocytes in which signals to initiate specific immunity are generated.

MMCs increase in size and quantity in fish in relation to a variety of physiological and environmental stressors and for this reason (high sensitivity and low specificity) they are used as a reliable biomarker for the state of fish health and for the quality of the aquatic environment (Steinel & Bolnick, 2017; Stosik et al., 2019).

In Agnatha and Chondrichthyes, MMs tend to be solitary or present in small aggregations of less than 30 cells (Wolke, 1992).

MMs in sharks have been investigated by only very few studies and, before using them as a first-line indicator, baseline levels have to be assessed.

Given their long life expectancy and life histories that predispose them to the accumulation of environmental toxins, sharks are believed to be good candidates as bioindicators of the health of the environment they live in (Borucinska et al., 2009; Gajić et al., 2020).

1.7 Lipid metabolism

Lipids are one of the four main classes of biological macromolecules. They are insoluble in water but soluble in organic solvents such as ether, alcohol, acetone and chloroform.

This class comprises an heterogeneous group of molecules, some are esters and have fatty acids as carboxylic acids, and some are hydrocarbons.

The base units of lipid esters are aliphatic chains of different lengths and degrees of saturation with a terminal carboxylic group known as fatty acids and these molecules have 4 main functions: they are stored as triglycerides in adipocytes to be used as energy reserves; they are the main components of phospholipids and glycolipids which are the lipids that compose biological membranes; they bind to membrane proteins to direct them in the right location; they are used as hormones and second intracellular messengers.

Sharks do not have a swim bladder and they use the liver as their hydrostatic organ together with all its other functions and it is also the principal site of lipid storage, therefore their hepatosomatic index (HSI) is higher compared to teleostean fish.

Shark liver's oil (SLO) is composed of triglycerides, which represent the main fraction, alkylglycerols and hydrocarbons, with squalene being the most represented (Davidson & Cliff, 2002; García et al., 2006; Jayasinghe et al., 2003; Navarro-Garcia et al., 2000; Sargent et al., 1973). Triglycerides are used as a fuel to supply the energy needed for metabolism while the others are mostly used for buoyancy since they have a lower density.

The lipid content of livers and the quality of fatty acids depends on the season, diet, sex, and habitat conditions: deep-sea species have more monounsaturated fatty acids, especially oleic acid (C18:1 n-9), as an adaptation to maintain a correct membrane fluidity in an environment with high pressure and low temperature (Remme et al., 2006); the salmon shark *Lamna ditropis* have a lipid content 20% higher in winter because in this season they feed on fish with high lipid content such as salmon, albacore tuna and other small elasmobranchs (Jayasinghe et al., 2003).

Studies on shark liver's oil (SLO) have been conducted mainly on deep-sea species and nowadays the focus is on how it may be used in pharmaceutical, medical and nutritional fields (García et al., 2006; Laurino & Palmieri, 2017) since it has antioxidant, anti-inflammatory, antibacterial, antifungal, antitumor properties given by its components.

1.8 Contaminants

Persistent organic pollutants (POPs), heavy metals, crude oil and marine debris, such as litter and microplastics (MPs), represent the most common marine pollutants globally and can reach the marine environment both from direct discharge and atmospheric transport.
Many pollutants bioaccumulate and biomagnify and apex predator tend to have high concentration of pollutants compared to environmental levels. Despite this, less attention has been paid to pollutants in elasmobranchs compared to other vertebrate groups both for the difficulties related to sampling and because fundings may support projects on species that have a more positive public perception.
Most of the studies on pollutants in elasmobranchs are focused on trace elements such as mercury (Hg), cadmium (Cd) and POPs such as PCBs and DDTs and have found that Hg is accumulates more in the muscle tissue while Cd, PCBs and DDTs accumulates in the liver (Tiktak et al., 2020).

The nature of the molecules determines where they accumulate: POPs are lipophilic molecules and therefore they are found in tissues with high lipid content such as the liver in sharks. POPs (PCBs and DDTs) are found also in ova and eggs: during egg-yolk formation females transfer hepatic lipids to maturing oocytes via a lipoglycophosphoprotein called vitellogenin, in a process known as vitellogenesis and contaminants are expected to passively follow hepatic lipids as they are mobilized. The positive relationship found between ova weight and contaminant load supports this statement (Lyons & Lowe, 2013) and the process of transfer from the mother to the embryo is called maternal offloading. The degree of chlorination of these molecules influences their lipophilicity and thus the ability to be transfered: less chlorinated PCB congeners are transfered more easily and embryos have higher proportions of DDTs metabolites with 4 chlorines and tri, tetra and penta PCBs congeners. This pattern is observed in marine mammals like the bottlenose dolphin (*Tursiops truncatus*) and ringed seals (*Phoca hispida*).

These molecules have a toxic effect because they can interfere with the metabolism of the animals: they can induce systems like the cyt-P450 which is involved in the synthesis of steroids and fatty acids and act as a competitive inhibitor; they can activate genes involved in other processes like cell proliferation and apoptosis; they can stimulate ROS formation. Furthermore, since they can be transfered to the embryo and can mimic the structure of steroid hormones, they can interfere with fetal development.

Mercury enters the marine environment in inorganic form and is transformed in its organic form, metilmercury, by sulphate-reducing bacteria in anoxic marine sediments and can enter the trophic net. It is bioaccumulated in the muscle tissue and it is the only metal that can be biomagnified: the highest the trophic level and the dimensions of the animal the higher is the level of mercury found in the muscle tissue.

Methylmercury toxicity in humans is related to the nervous system both in adults and in embryos since it can be offloaded by the mother. Methylmercury can be offloaded to elasmobranch embryos as well but the effects on their physiology is not known (北村 et al., 1997). Furthermore, it has been observed that tolerance to pollutants is different among elasmobranch species but the effects have been more focused on risks related to humans who consume shark derived products rather than the animals themselves (Tiktak et al., 2020).

Microplastics (MPs) are defined as fragments of less than 5mm in dimension of various shape and nature. The main components of MPs are heavily produced low density plastics such as polypropylene, polystyrene and polyethylene.

MPs are either discharged as such since are used as pellets in the plastic industry or as abrasives, or can derive from bigger items that undergo a process of degradation and breakdown.

Due to their low weight, MPs can be transported by currents for long distances and are found worldwide in the marine environment and are considered carriers of POPs due to the high affinity of hydrophobic organic chemicals for MPs.

MPs constitute a great environmental concern and have negative effects on marine fauna because they can transport pollutants; additives such as phtalates can leach from the plastic; and the particles can cause physical harm to the animals (Cristina et al., 2020).

MPs in elasmobranchs have not been widely studied and the few articles on this topic do not take in consideration MPs effects on the liver of these animals.

In conclusion, elasmobranch liver are largely composed of neutral lipids with a storage function such as triacylglycerols, which differ from the more polar lipids found in other tissues such as the nervous and muscular ones. Thus, lipid content and composition probably play a role in how contaminants partition themselves among tissues.

Elasmobranchs are understudied from a toxicological point of view and this study tries to shed new light on these topics.

Chapter 2

AIM OF THE PROJECT

The aim of this project is to identify new biomarker for the evaluation of the health status of sharks caught in the Adriatic Sea and their susceptibility to environmental changes.

To reach this objective, melanomacrophages have been used as stress biomarkers on these organisms, since they are already widely used in teleosts, amphibians and reptiles.

In this study, hepatic melanomacrophages have been evaluated in four species of sharks caught in the Adriatic Sea, both from a morphological and quantitative point of view, evaluating differences among species, sexes and, where possible, between these and the reproductive period. In conclusion, it has been assessed if there was any significant correlation between melanomacrophages and age of smooth-hound males.

Chapter 3

MATERIAL AND METHODS

2.1 Sampling

The animals were collected by fishing vessels in the Adriatic Sea (FAO Geographical Area 37, Subarea 37.2, Division 37.2.1) from January 2021 to May 2021. Total length was measured from the tip of the snout to the upper lobe of the caudal fin and total body weight was measured to the nearest gram. At autopsy, liver weight and gonadal weight were measured and two sets of random samples of liver and gonad were collected: one set was fixed in Formol for histology and the other stored at -80°C for cryostat and FT-IR analysis.

2.2 Indices

Gonadosomatic index

The gonadosomatic index [%] was determined using the formula

$$GSI = G_W / W_T \cdot 100$$

where G_W is the gonad weight (g), and W_T the total weight of fish (g) (Bagenal, 1978).

Hepatosomatic index

The hepatosomatic index [%] was determined using the formula

$$HSI = H_W / W_T \cdot 100$$

where H_W is the hepatic weight (g), and W_T the total weight of fish (g) (Capapé & Reynaud, 2011).

Condition factor

Condition factor was determined using the formula

$$K = (W_T / L_T) \cdot 10^{-3}$$

Where W_T is the total weight of fish (g) and L_T the total length of fish (m) (Blackwell et al., 2000).

2.3 Histology

- *Paraffin inclusion*

  Samples fixed in Formol underwent the embedding process: the first step consists of placing samples into biocassettes and removing fixative and water through a series of ascending graded ethanol baths. After dehydration, the protocol used required a clearing process through xylene baths, to remove the dehydrating medium and to prepare the samples for the final embedding process which required samples to be placed in paraffin wax.

- *Microtome*

  Samples embedded in paraffin blocks were cut into sections using the microtome, with a thickness of 5µm. Sections were put in a bath of lukewarm deionized water before being placed on a glass slide. For each slide, a total of 3 sections per sample were placed. Glass slides were left drying overnight at room temperature for the sections to adhere completely to the surface of the slide before proceeding with the staining.

- *Staining process*

  Sections were stained with Hematoxylin & Eosin. At first, this protocol requires the samples to be deparaffinized through a consequential bath of xylene (Xylene 1 and Xylene 2) before proceeding with a series of decreasing graded ethanol baths (100%, 95%, 80%, 70%). After that, the slides were soaked first in Hematoxylin, rinsed in a bath with running tap water to remove the excess dye, then in Eosin and rinsed again. The stained slides were then placed in a series of ascending graded ethanol baths (70%, 80%, 95%, 100%) and the last bath in xylene to facilitate the mounting of a coverslip on the slide under the chemical hood.

After some trial, a soaking time of 75 seconds in Hematoxylin and 30 seconds in Eosin were chosen, to have clearer staining on sections of both liver and gonads.

- *Optical microscope*

  Stained sections were examined under Zeiss microscope and 2 random high-power fields (HPF, 40x) per liver section were selected for a total of 6 HPF in total per sample.

- *ImageJ*

  Obtained HPFs were analyzed with imaging software ImageJ and mean number of HPF, mean surface area of HPF, mean percentage area of HPF covered by MMs and MMCs, and mean percentage area covered by lipids were calculated.

  The criteria used for counting MMs and MMs were:

  1) The cytotype has to be evident: interspersed pigmented granules were not considered in the count.
  2) If the pigmented cell had a circular shape and a homogenous coloration was considered as MM, on the contrary, it was considered as MMC.
  3) Clusters of pigmented granules delimited by a membrane and through which the color of the hepatocyte cytoplasm was visible were not included in the count.
  4) MMs/MMCs cut by the frame of the image were not included in the count.

The protocol for calculating the surface area of MMs and MMCs and the percentage area covered by them was:

Open image in ImageJ > Select “Straight line” and draw a line of the same length of the scale bar > Go to “Analyze” > “Set Scale” > Type “20” in “Known distance” and “um” in “Unit of length” > Select “Global” and press “OK”

Go to “Analyze” > “Set Measurements” > Select “Area” and “Area fraction” and press “OK”

Select “Freehand selections” > Outline the MM or the MMC > Go to “Analyze” > “Measure”

For lipid quantification the protocol was as follows:

Open image in ImageJ > Go to “Image” > “Type” > “8-bit”

Go to “Analyze” > “Set Measurements” > Select “Area fraction” only and press “OK”

Go to “Image” > “Adjust” > “Threshold” > Adjust the threshold until all the lipid globules (white areas) are highlighted and nothing else > Go to “Analyze” > “Measure”

2.4 Statistical analysis

Statistical analysis was performed using GraphPad software for ANOVA and t-test analysis and JASP software was used for Pearson’s correlation analysis. One-way ANOVA followed by Tukey’s post hoc test was performed to evidence differences of all melanomacrophages and lipids parameters among species and sexes. Two tailed t-test was performed to evidence differences between males and females of *M.mustelus* both in pre and post-mating season. Pearson’s correlation was performed among all biometric, melanomacrophages and lipids parameters of males of *M.mustelus.*

2.5 FT-IR measurements and data analysis

A Bruker VERTEX 70 interferometer coupled with a Hyperion 3000 Vis-IR microscope was used. The spectrometer was equipped with a liquid nitrogen cooled bidimensional Focal Plane Array (FPA) detector that allows to perform the imaging analysis of non-homogeneous biological samples by simultaneously acquiring 4096 spectra on an area of 164x164 μm2. The visible image of each liver section was obtained with a 15X condenser/objective. On these selected areas, IR maps were collected in transmission mode in the 4000-900 cm^{-1} MIR range with a spatial resolution of ~2.56 μm. Each spectrum was the result of 256 scans with a spectral resolution of 4 cm^{-1}. Background spectra were acquired on clean regions of CaF2 optical windows.

Raw IR maps were corrected by applying the Atmospheric Compensation routine, to remove the contribution of atmospheric carbon dioxide and water vapour, and then vector normalized in the 4000-900 cm-1 spectral range, to avoid artefacts due to differences in thickness (OPUS 7.1 software, Bruker Optics, Ettlingen, Germany).

These preprocessed IR maps were integrated under the following spectral regions, to obtain false colour images representing the topographical distribution and relative amount of the most relevant biochemical features: 2995–2825 cm^{-1} (containing the vibrational modes of lipids, named LIPIDS); 1718–1481 cm^{-1} (containing the vibrational modes of proteins, named PROTEINS); 1274–1181 cm^{-1} (containing the vibrational modes of phosphates groups inside nucleic acids, named PHI DNA), and 1130–1013

cm^{-1} (containing above all the vibrational modes of pentos, named PHII DNA). An arbitrary colour scale was used, white colour indicating areas with the highest absorbance values and blue colour areas with the lowest ones.

Chapter 4

RESULTS

The presence of melanomacrophages in liver sections was determined in the samples belonging to four species of shark: the blue shark, *P.glauca* (PG); the small-spotted catshark, *S.canicula* (SC); the spiny dogfish, *S.acanthias* (SA)*;* and the common smooth-hound *M.mustelus* (MM). By comparing morphology and color of melanomacrophages, differences between the species emerged (Fig.1). Very few melanomacrophages were found in the blue shark and they appear regular in shape and homogenous in color (Fig.1a). In the small-spotted catshark, melanomacrophages were found in high number and big in size, tending towards a rounded shape, both with a grainy texture and homogenous in color (Fig.1b). In the spiny dogfish these cells appear irregular in shape and color (Fig. 1c). In smooth-hounds they tend to assume a regular shape and a homogenous coloration. Some centers are surrounded by a capsule, which represents a superior level of organization (Fig.1d).

From liver sections it was possible to qualitatively analyze the lipid globules present in the tissue. Differences between species regarding lipid globules were found (Fig.1). In the blue shark's section all lipid globules have a rounded shape and two types of lipid globules, based on dimensions, were found: one with a medium diameter of 64,230 µm and one with much smaller globules than the former, with a medium diameter of 17,538 µm. The big lipid globules do not tend to merge and they remain well separated from each other (Fig.1a) In the small-spotted catshark, lipid globules are evident but small (Fig.1b). In the spiny dogfish, all lipid globules have a rounded shape and have a similar size of around 28.026 µm in diameter. Some globules tend to merge while others stay separated (Fig.1c). In the smooth-hound the dimensions of the globules are variable and the shape is not properly rounded due to the merging of more globules (Fig. 1d).

Macromolecular characterizations of the liver sections are shown in Figure 2-5. The white areas in the microphotographs correspond to the highest absorption values of lipids while the areas with the higher absorption values of proteins corresponds to the parenchymatous hepatic tissue.

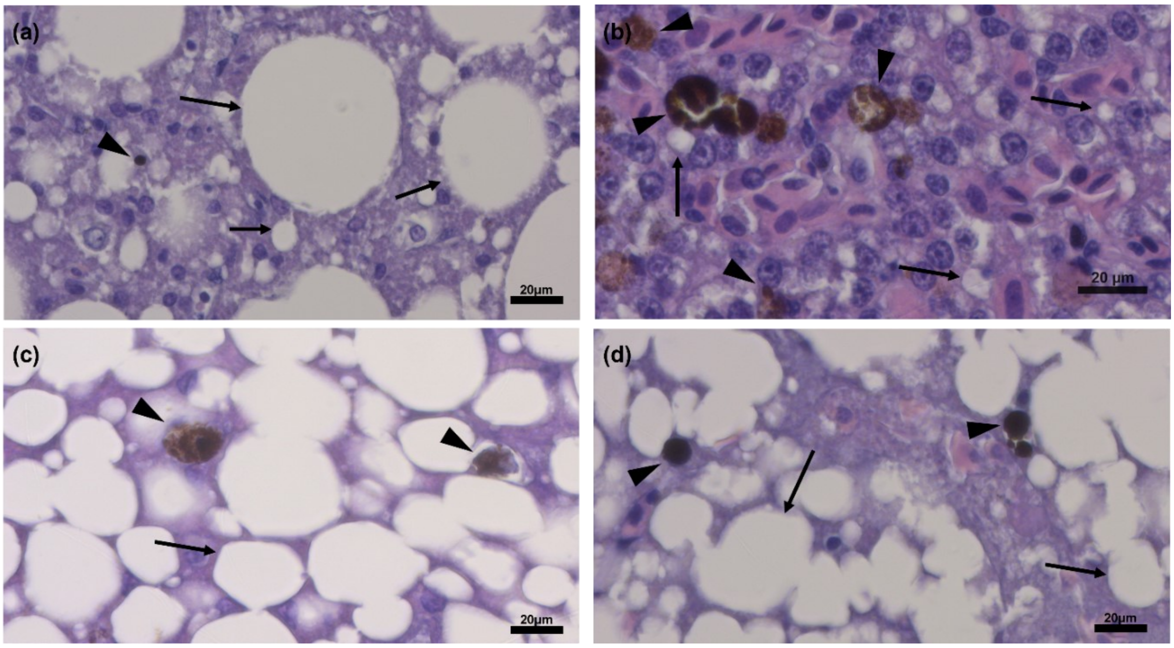


Figure 1 *– Liver's section of (a) blue shark, P.glauca; (b) small-spotted catshark, S.canicula; (c) spiny dogfish, S.acanthias; (d) common smooth-hound; M.mustelus. Arrows indicate lipid globules, arrowheads indicate melanomacrophages. Hematoxilin and eosin, 40x.*

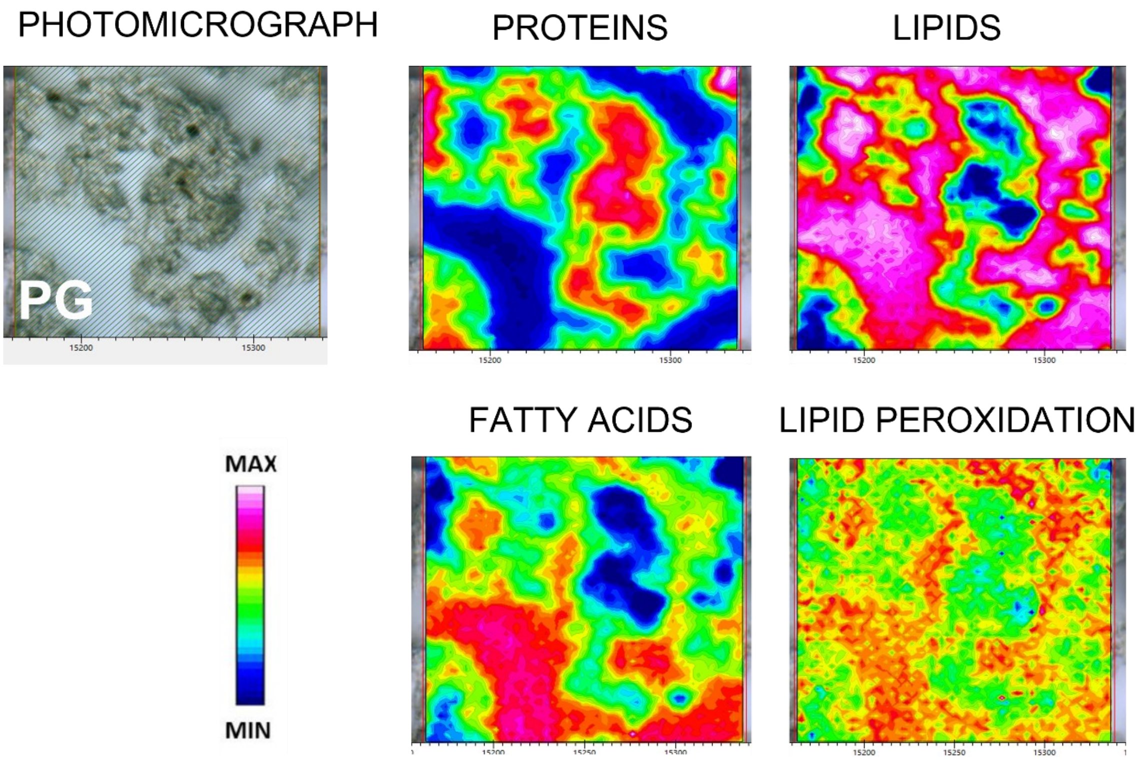


Figure 2 *- FT-IR microspectroscopy analysis of a liver section of P.glauca. Microphotograph (164x164 µm2). IR maps representing the topographical distribution of: Proteins, Lipids, Fatty Acids and Lipid Peroxidation relative to unsaturated fatty acids. Due to different molar extinction coefficients of the analyzed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).*

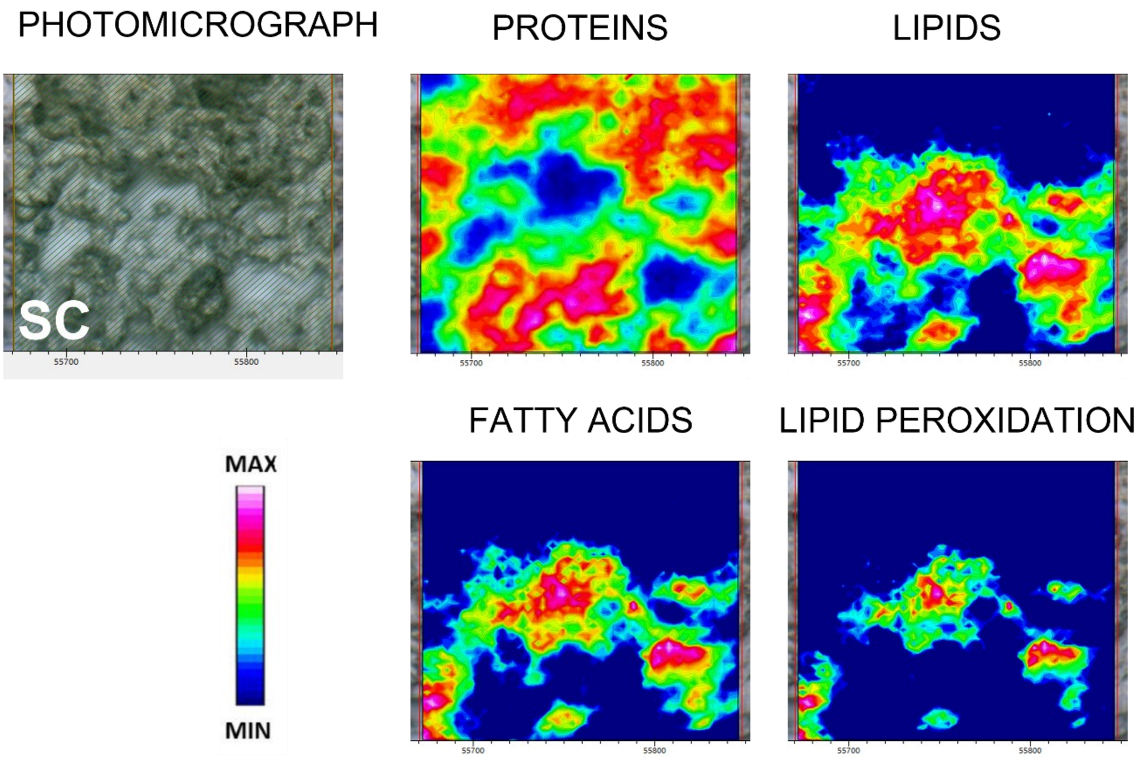


Figure 3 - *FT-IR microspectroscopy analysis of a liver section of S.canicula. Microphotograph (164x164 µm2). IR maps representing the topographical distribution of: Proteins, Lipids, Fatty Acids and Lipid Peroxidation relative to unsaturated fatty acids. Due to different molar extinction coefficients of the analyzed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).*

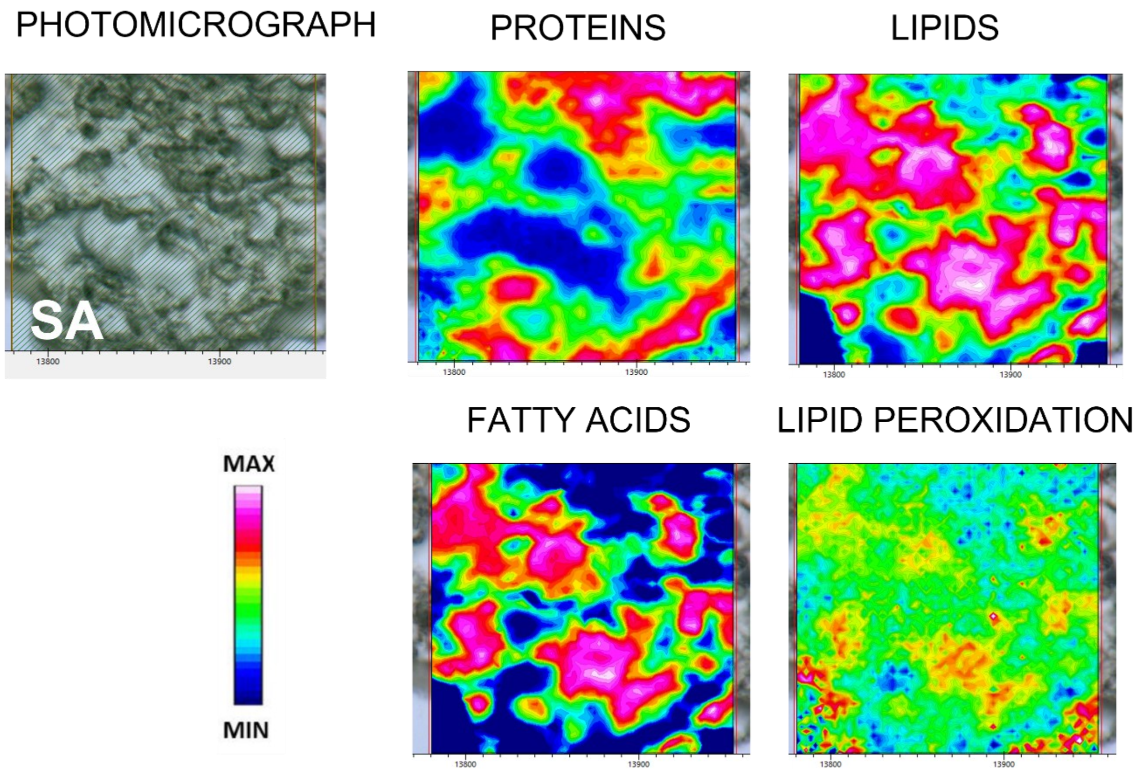


Figure 4 *- FT-IR microspectroscopy analysis of a liver section of S.acanthias. Microphotograph (164x164 μm2). IR maps representing the topographical distribution of: Proteins, Lipids, Fatty Acids and Lipid Peroxidation relative to unsaturated fatty acids. Due to different molar extinction coefficients of the analyzed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).*

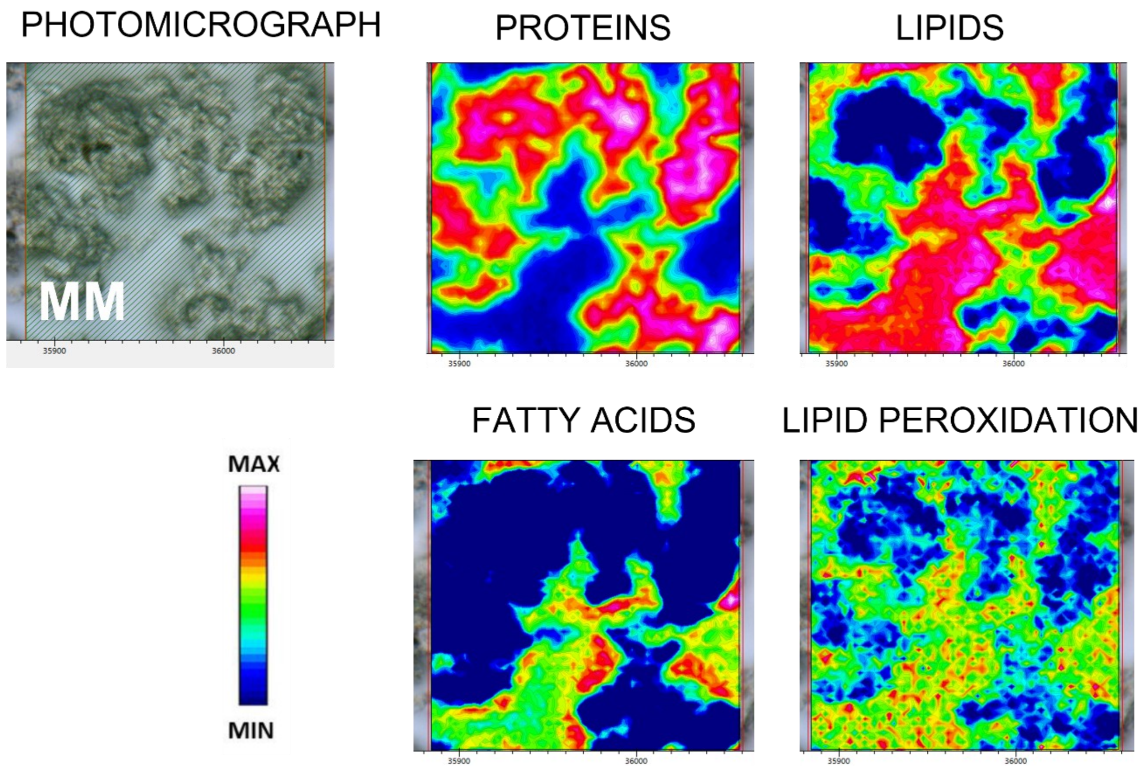
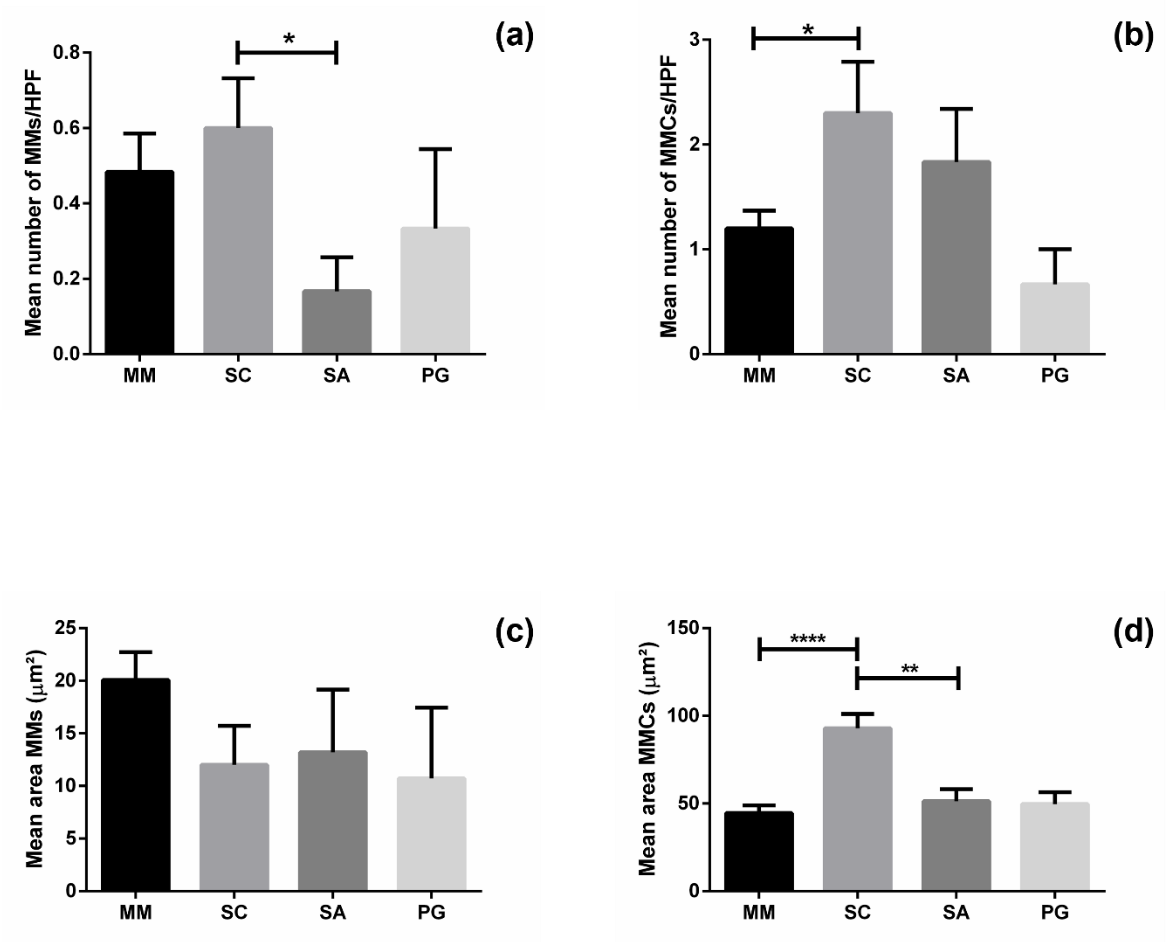


Figure 5 *- FT-IR microspectroscopy analysis of a liver section of S.acanthias. Microphotograph (164x164 µm2). IR maps representing the topographical distribution of: Proteins, Lipids, Fatty Acids and Lipid Peroxidation relative to unsaturated fatty acids. Due to different molar extinction coefficients of the analyzed peaks, different scales were used for each IR map (blue colour indicating the areas with the lowest absorption values, while white colour the highest ones).*

Figure 6 shows the differences between the mean number and mean area of melanomacrophages (both MMs and MMCs) among species. Figure 6a shows a significant difference of mean number of MMs between SC and SA ($p < 0.05$). Regarding the mean number of MMCs, a statistically significant ($p < 0.05$) difference was evidenced between MM and SC (Fig. 6b). No differences were evidenced among species about mean area of MMs (Fig. 6c). Concerning the mean area of MMCs, statistically significant differences were found between MM and SC ($p < 0.0001$) and between SC and SA ($p < 0.01$) (Fig. 6d). No differences were found about the mean percentage of HPF occupied by MMs among species (Fig. 6e). Regarding the mean percentage of HPF occupied by MMCs, statistically significant differences were evidenced between MM and SC ($p < 0.0001$) and between SC and SA ($p < 0.01$) (Fig. 6f).


(a)
Mean number of MMs/HPF
0.0
0.2
0.4
0.6
0.8
*
MM
SC
SA
PG
(b)
Mean number of MMCs/HPF
0
1
2
3
*
MM
SC
SA
PG
(c)
Mean area MMs (μm²)
0
5
10
15
20
25
MM
SC
SA
PG
(d)
Mean area MMCs (μm²)
0
50
100
150
****
**
MM
SC
SA
PG

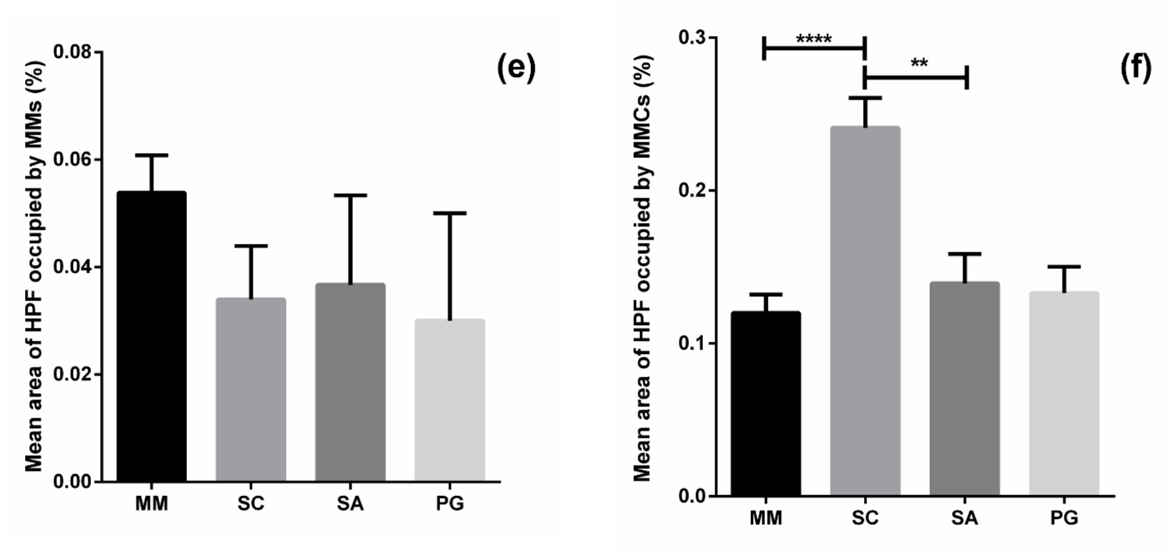


Fig.6 – *Histogram shows mean number (a, b), mean area (c, d) and mean percentage of HPF occupied by MMs and MMCs (e, f) (mean ± SEM). MM (M.mustelus); SC (S.canicula); SA (S.acanthias); PG (P.glauca) (p-value < 0.05).*

Regarding hepatic lipid content, the lowest amount was found in SC while the highest amount in SA. MM and PG showed intermediate values (Fig.7).

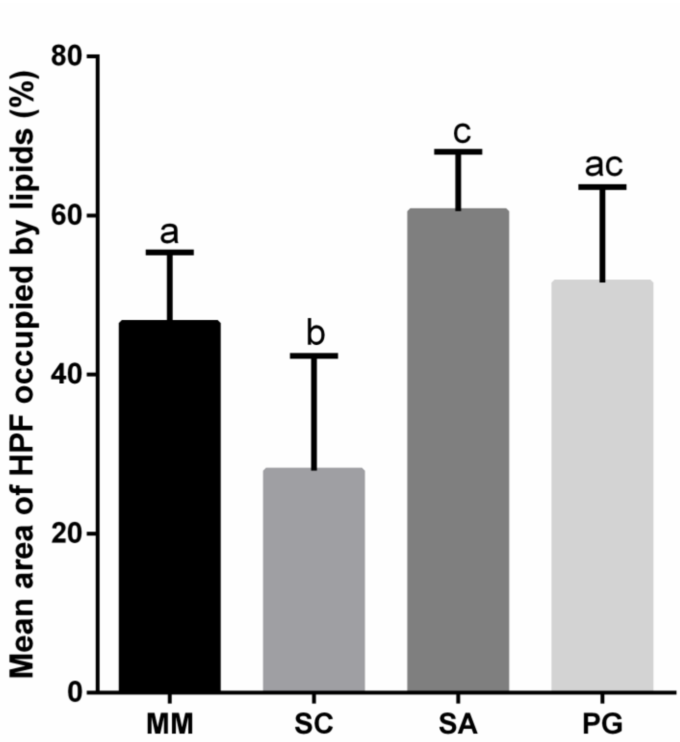
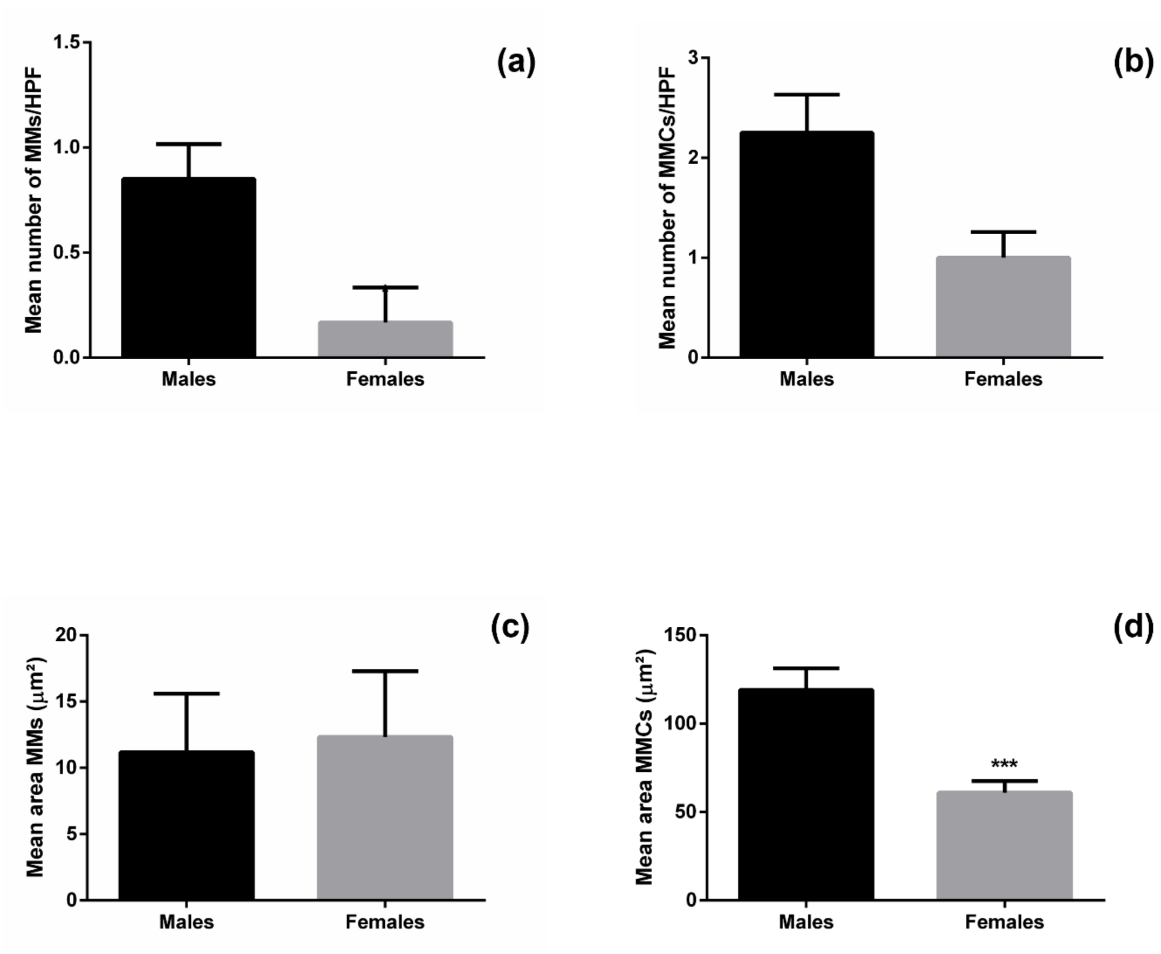


Fig.*7 - Histogram shows levels of hepatic lipid content in HPF (mean ± SEM). MM (M.mustelus); SC (S.canicula); SA (S.acanthias); PG (P.glauca). Different letter indicates statistically significant differences.*

Where there was the availability of males and females, a comparison was made between melanomacrophages and lipids quantification among sexes. In SC, females showed the lowest mean number of MMs compared to males ($p < 0.05$) (Fig. 8a). A similar trend was observed for mean number of MMCs, but no statistically significant differences were evidenced (Fig. 8b). Concerning mean area of MMs no statistically significant differences were found between sexes (Fig. 8c). Conversely, statistically significant differences were evidenced focusing on mean area of MMCs, where males showed higher values ($p < 0.001$). No differences were evidenced between sexes about mean percentage of HPF occupied by MMs (Fig. 8e). Regarding mean percentage of HPF occupied by MMCs, higher values were found in males ($p < 0.001$) (Fig. 8f).



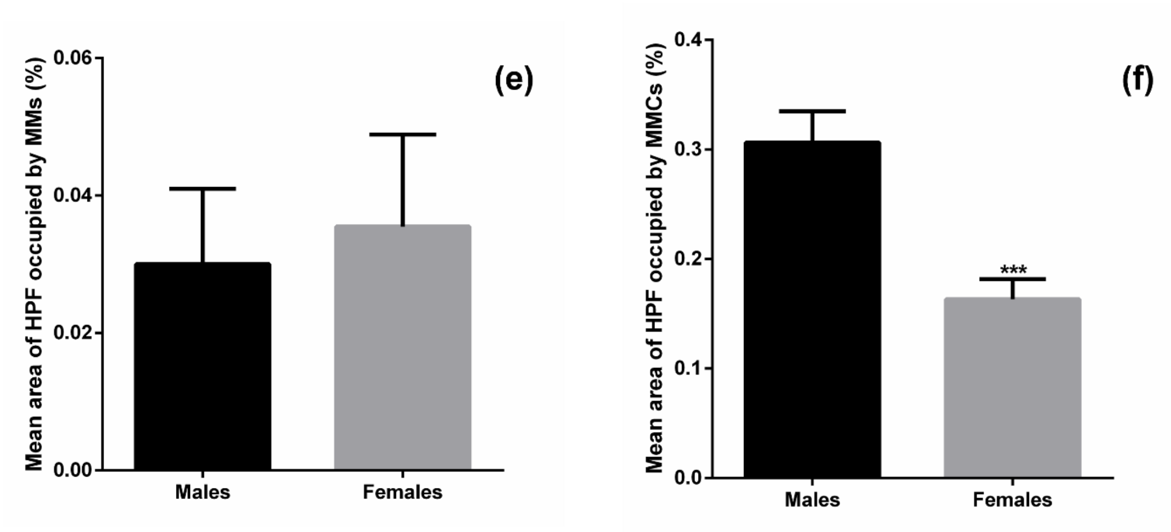


Fig. 8 - *Histogram shows mean number (a, b), mean area (c, d) and mean percentage of HPF occupied by MMs and MMCs (e, f) in males and females of S.canicula (mean ± SEM) (p-value < 0.001).*

Concerning hepatic lipid content, females showed a significantly higher amount in females rather than males ($p < 0.001$) (Fig.9).

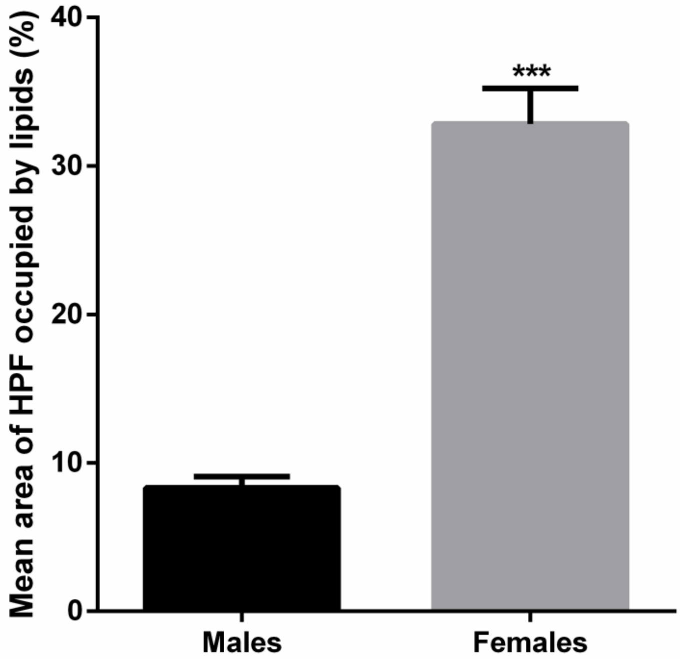
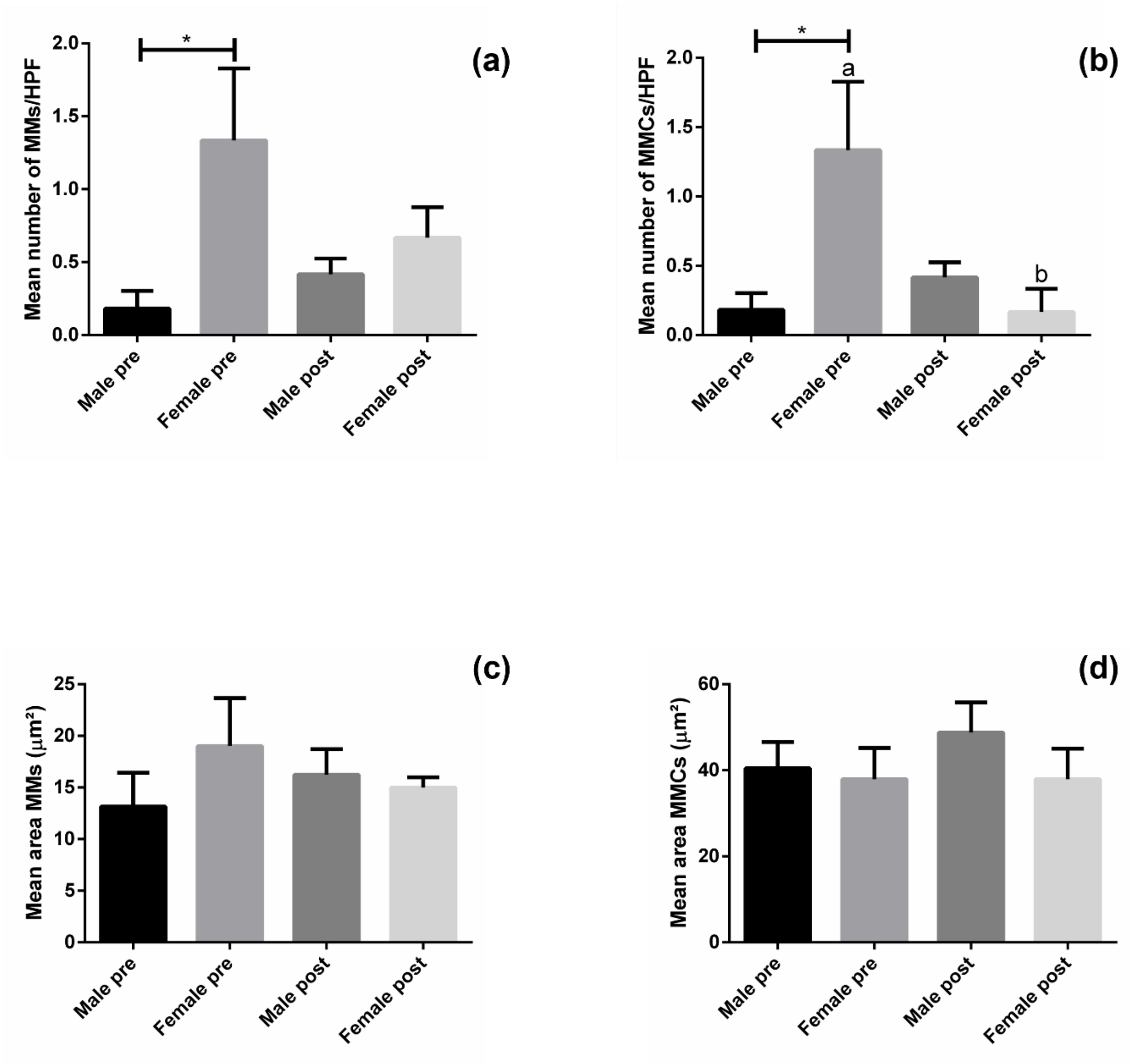
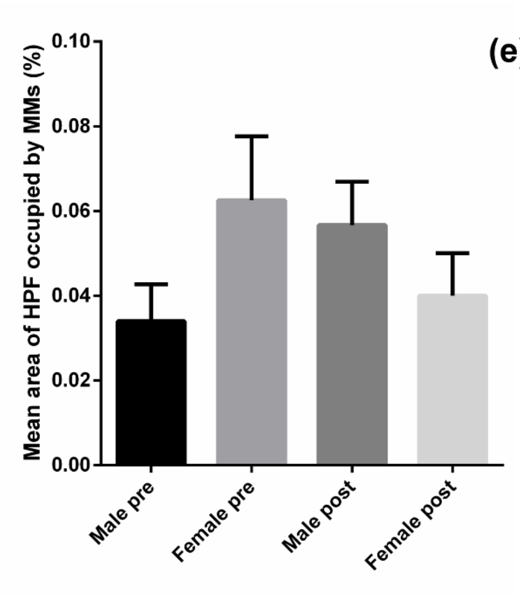


Fig. 9 - *Histogram shows levels of hepatic lipid content in HPF in males and females of S.canicula (mean ± SEM) (p-value < 0.001).*

In MM, individuals of both sexes before and after the mating season were available. Differences were evidenced about mean number of MMs between males and females pre-mating ($p < 0.05$), while, in the post-mating season, an increasing trend in the former and a decreasing trend in the latter with no statistically significant relevance were found (Fig. 10a). Concerning the mean number of MMCs, differences were found between males and females pre-mating ($p < 0.05$) and between females in the pre-mating season and females in the post-mating season ($p < 0.05$). Between males pre and post-mating, an increasing trend is visible but with no statistical significance (Fig. 10b). No differences were evidenced about mean area of MMs, mean area of MMCs, mean percentage of HPF occupied by MMs and mean percentage of HPF occupied by MMCs in both sexes and in both seasons (Fig. 10c-f).





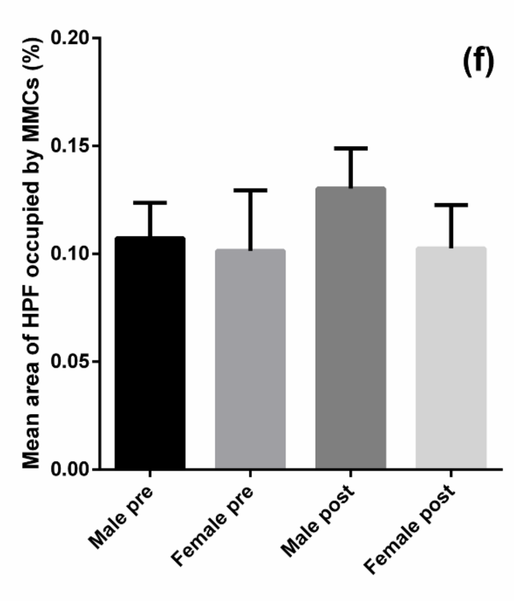


Fig.10 - *Histogram shows mean number (a, b), mean area (c, d) and mean percentage of HPF occupied by MMs and MMCs (e, f) in males and females of M.mustelus in pre and post-mating season (mean ± SEM) (p-value < 0.05).*

Regarding hepatic lipid content, statistically significant differences were evidenced between males pre and post-mating ($p < 0.05$), between females pre and post-mating ($p < 0.0001$), between males and females pre-mating ($p < 0.0001$) and between males and females post-mating ($p < 0.0001$) (Fig. 11).

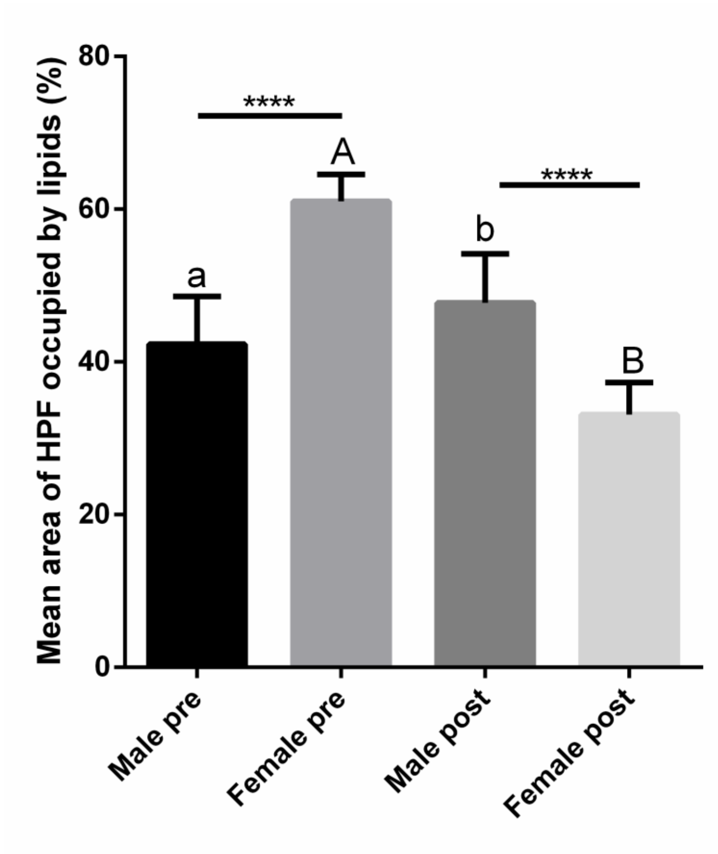


Fig.11 - *Histogram shows levels of hepatic lipid content in HPF in males and females of M.mustelus in pre and post-mating season (mean ± SEM). Different letter indicates statistically significant differences (p-value < 0.0001).*

In males of MM correlations between all biometric data and data on melanomacrophages and lipids were found (Table 1).

.

Variable		Lt(m)	Wt(g)	Hw(g)	Gw(g)	GSI	HSI	K	Mean n°MMs	Mean n°MMC	Mean area MMs	Mean area MMCs	Mean % MMs on HPF	Mean % MMCs on HPF	% lipids in HPF
1. Lt(m)	Pearson's r	—													
	p-value	—													
2. Wt(g)	Pearson's r	**0.955**	—												
	p-value	*< .001*	—												
3. Hw(g)	Pearson's r	**0.884**	**0.939**	—											
	p-value	*0.004*	*< .001*	—											
4. Gw(g)	Pearson's r	**0.820**	**0.725**	**0.786**	—										
	p-value	*0.013*	*0.042*	*0.021*	—										
5. GSI	Pearson's r	0.510	0.347	0.462	**0.893**	—									
	p-value	*0.197*	*0.400*	*0.249*	*0.003*	—									
6. HSI	Pearson's r	**0.745**	**0.752**	**0.921**	**0.818**	0.632	—								
	p-value	*0.034*	*0.032*	*0.001*	*0.013*	*0.093*	—								
7. K	Pearson's r	**-0.916**	**-0.773**	**-0.725**	**-0.811**	-0.634	-0.696	—							
	p-value	*0.001*	*0.024*	*0.042*	*0.015*	*0.091*	*0.055*	—							
8. Mean n°MMs	Pearson's r	-0.684	**-0.810**	**-0.769**	-0.346	0.011	-0.589	0.528	—						
	p-value	*0.061*	*0.015*	*0.026*	*0.401*	*0.980*	*0.124*	*0.179*	—						
9. Mean n°MMC	Pearson's r	0.633	0.586	0.460	0.649	0.543	0.379	-0.625	-0.305	—					
	p-value	*0.092*	*0.127*	*0.252*	*0.081*	*0.165*	*0.355*	*0.097*	*0.462*	—					
10. Mean area MMs	Pearson's r	-0.310	-0.428	-0.313	0.104	0.462	-0.057	0.029	0.336	0.187	—				
	p-value	*0.454*	*0.290*	*0.451*	*0.806*	*0.249*	*0.894*	*0.945*	*0.415*	*0.657*	—				
11. Mean area MMCs	Pearson's r	0.269	0.264	0.421	0.526	0.551	0.604	-0.317	-0.040	0.581	0.356	—			
	p-value	*0.519*	*0.527*	*0.299*	*0.180*	*0.157*	*0.112*	*0.445*	*0.925*	*0.131*	*0.387*	—			
12. Mean % MMs on HPF	Pearson's r	-0.310	-0.428	-0.313	0.104	0.462	-0.057	0.029	0.336	0.187	**1.000**	0.356	—		
	p-value	*0.454*	*0.290*	*0.451*	*0.806*	*0.249*	*0.894*	*0.945*	*0.415*	*0.657*	*< .001*	*0.387*	—		
13. Mean % MMCs on HPF	Pearson's r	0.285	0.281	0.442	0.546	0.568	0.626	-0.328	-0.053	0.581	0.346	**0.999**	0.346	—	
	p-value	*0.494*	*0.501*	*0.273*	*0.161*	*0.142*	*0.097*	*0.427*	*0.901*	*0.131*	*0.401*	*< .001*	*0.401*	—	
14. % lipids in HPF	Pearson's r	-0.357	-0.497	-0.347	-0.190	0.104	-0.027	-0.005	0.314	-0.177	0.624	0.300	0.624	0.287	—
	p-value	*0.386*	*0.210*	*0.399*	*0.652*	*0.806*	*0.949*	*0.990*	*0.448*	*0.674*	*0.098*	*0.470*	*0.098*	*0.491*	—

Tab 1 *– Pairwise analysis of Pearson' s correlation test. Bold values are considered significative for a p-value < 0.05. GSI = Gonadosomatic index; HSI = Hepatosomatic index; K = Condition factor.*

Chapter 5

DISCUSSIONS

Sharks are considered endangered by overfishing because of their life history traits, but is this the only threat these animals are facing or environmental changes are also playing a role in their overall health status?

In order to determine the environmental stress, biomarkers are needed and melanomacrophages have been used in a wide range of species of fish (Kalita et al., 2019; Nowak et al., 2021; Qualhato et al., 2018; Viana et al., 2021) while in sharks these cells have been poorly studied. To be able to use these cells as a valuable biomarker the first step is understanding how they work and if there are any variations not related to the environmental stress, such as variations among sexes, due to the reproductive period and to age. In this study melanomacrophages were observed for the first time in the liver of *M.mustelus* and *S.acanthias* while in *P.glauca* they were observed by Borucinska et al., 2009 in the northwestern Atlantic and in *S.canicula* by Gajić et al., 2020 in the eastern Adriatic.

The results show that *S.canicula* have the highest number of both MMs and MMCs, an higher mean area of MMCs as well as a lower hepatic lipid content compared to the other considered species. Data on morphometric values of melanomacrophages suggest that this species may be more subjected to acute and chronic stress compared to the other species considered (Agius & Roberts, 1981; 2003; Kalita et al., 2019; Qualhato et al., 2018; Viana et al., 2021; Wolke, 1992). This may be explained by the fact that this species is a demersal species closely related to the bottom, where lipophilic pollutants tend to accumulate. Due to the contribution of Po River, the Adriatic Sea is characterized by fine grain sediments, from sand to silt to clay moving outward from the coast (Palumbo & Selvaggi, 2003; SNPA, 2018) and the lowest the grain size of the sediment, the higher is the adsorption capacity of it (Karickhoff et al., 1979). Therefore bottom-dwelling species are more exposed to contaminants that accumulate in the sediments. Furthermore, as observed in other studies, sharks tend to accumulate more lipophilic contaminants in the liver due to a higher content of lipids and a lower activity of cytochrome P450 enzymes compared to teleosts (Cresson et al., 2016; Gorbi et al., 2004; Jeffree et al., 2006, 2010). Moreover, small-spotted catshark shows a strong genetic differentiation in the Mediterranean basin due to a high degree of site fidelity and a low dispersal capacity (Rodríguez-Cabello et al., 2004; V. Kousteni et al., 2015) therefore it might be more susceptible to long-term pollution. According to Combi et al., 2020 , PAH, PCBs and DDTs in the sediments of the western Adriatic Sea pose limited risk of toxicity in marine organisms, except for the PAH dibenzo[a,h] anthracene. However, their study did not take in consideration other pollutants such as microplastics or heavy metals.

From the results of this study, hepatic lipid content is around 3.5 times higher in females rather than males of *S.canicula*. Annual variation in HSI in the small-spotted catshark occur both in males and in females but are wider in the latter because continuous vitellogenesis occurs (Craik, 1978). The variation of this parameter in both sexes is related to deposition of lipid reserves in the liver based on season: in the Adriatic and Aegean Sea it has been observed that feeding intensity is lower in winter due to a decrease in food availability, metabolic rhythm and thus food demands (Kousteni et al., 2017; Jardas et al. 2004; Šantić et al. 2012). An aspect that comes out from the results in this study is the fact that dimensions of MMCs in *S.canicula* are inversely proportional to hepatic lipid content suggesting that there might be a correlation between these two parameters in this species.

In *M.mustelus*, MMCs are present in lower numbers in the post-mating season in both sexes and they are always more abundant than MMs in males; in females, MMs are more abundant than MMCs in the pre-mating season while in the post-mating season the latter is present in higher number than the former. For what it concerns lipids, females have more hepatic lipid content than males in the pre-mating season while in the post-mating season males have more lipid in their liver rather than females.

For what concerns *M.mustelus*, females sampled in the pre-mating period have a mean number of both MMs and MMCs higher than males, suggesting that they may be more susceptible to both acute and long-term stress. However, females in post-mating period show a significant decrease in the mean number of MMCs and hepatic lipid content compared with female in pre-mating period. These results may suggest that females during their preparation to the mating season, invest their energy mainly towards reproductive functions, such as vitellogenesis, instead than towards the maintenance of the homeostasis. Furthermore, following this hypothesis, they would need more energy to accomplish vitellogenesis, resulting in a higher food intake and therefore a higher level of liver metabolism. *M.mustelus* is a demersal species that feeds mainly on benthic invertebrates and bony fishes therefore it might be exposed to lipophilic contaminants that accumulate in liver due to its high lipid content, similarly to *S.canicula*. *M.mustelus* is a viviparous species therefore, in addition to large yolk-filled eggs, viviparous elasmobranchs provide additional nutrition to embryos in the form of yolksac-placental conveyance (Wourms et al., 1988) and lipophilic pollutants can be transferred to the ova during vitellogenesis, and to the embryo during pregnancy (北村 et al., 1997; Tiktak et al., 2020; Lyons et al., 2021). The low hepatic lipid content might mean that vitellogenesis have occurred and that the decrease of number of MMCs is related or to a better functioning of the immune system after the mating period or to a detoxification process of the liver due to the offloading of pollutants to the ova.

To validate this hypothesis further research is needed and also it can not be excluded that the results obtained in this study are biased from sample availability.

Finally, no significant correlation between total length (L_T) and mean number of MMCs was found in males of *M.mustelus*. This result could be due to the fact that the individuals of all ages are equally impacted by some sort of stress or these individuals show a narrow age span for this species. In fact, these specimens have a range of L_T from 0.6m to 1.1m, corresponding to a range from 3 to 8 years (Ozcan & Başusta, 2018). To confirm these outcomes new specimens and analysis are needed.

In conclusion, this study is just a staring point which provides new solid insights on identifying new valuable biomarkers to monitor health status of sharks and their susceptibility to environmental changes.

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