UNIVERSITÀ POLITECNICA DELLE MARCHE

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Corso di Laurea Magistrale

Biologia Marina

Belli fuori, brutti dentro? Caratterizzazione dello stato di salute della *Sardina pilchardus* in relazione allo stato del fegato, crescita e sforzo di pesca

Pretty on the outside ugly on the inside? Characterization of the health status of *Sardina pilchardus* in relation to the state of liver, age growth and fishing effort

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A mio nonno, che mi ha fatto dono della curiosità.

A mio padre, che con i suoi sacrifici mi

ha permesso di seguirla.

A Greta, che giorno dopo giorno la

mantiene viva in me.

"Dritto alla meta e conquista la preda"

- *Capitan Jack Sparrow*

RIASSUNTO

La sardina, *Sardina pilchardus* (Walbaum, 1792), rappresenta un'importante risorsa sia dal punto di vista ecologico, per il suo ruolo all'interno della rete trofica, sia economico, per il suo valore commerciale. Inoltre, è una specie suscettibile alle variazioni delle condizioni ambientali e questo fa di lei un buon biomarker.

Data la scarsa quantità di informazioni che si ha sullo stato di salute della sardina, nonostante la sua importanza, questo studio si è posto l'obiettivo di indagare su tale aspetto tramite l'analisi dei melanomacrofagi e analisi istopatologica del tessuto epatico.

Ciò che ne è emerso è una situazione drastica. Molti individui presentano infezioni parassitarie che, nonostante non ne determinino un aumento, vengono riconosciute come no-self dai melanomacrofagi. Inoltre, l'analisi istopatologica ha messo in mostra come la quasi totalità degli individui presenti dei danneggiamenti al fegato, a diversi livelli di gravità. Ciò potrebbe dipendere dalla presenza di fattori di stress come cambiamenti climatici e inquinanti.

Questo studio, dunque, rappresenta la base per la realizzazione di un monitoraggio più rigoroso da condurre su base annuale.

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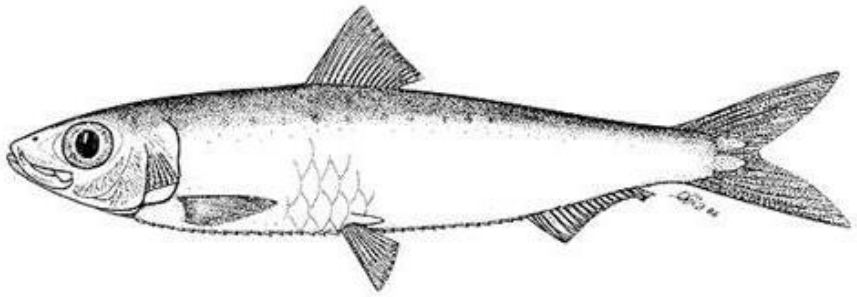
1. INTRODUCTION

1.1. Morphology and distribution of Sardina pilchardus

1.1.1. Morphology

European sardine (*Sardina pilchardus,* Walbaum,1792) is a small pelagic fish belonging to the family of Clupeidae. It, with other clupeids, is called “blue fish” for its coloration: the back is greenish-blue, while the belly is white; this for an anti-predatory action.

The sardine also has a quite elongated body, slightly compressed on the sides, with deciduous scales. The pelvic fin origin behind the dorsal fin, while the two ventral fins develop in correspondence with the middle of the dorsal fin, which is in the centre of the body [Fig.1]. This feature is important to discriminate the sardine from the European sprat *Sprattus sprattus* (Linnaeus, 1758). Instead, the morphology of its mouth is important to determine the difference with the anchovy *Engraulius encrasicolus* (Linnaeus, 1758). In fact, in the anchovy, the upper jaw is longer than the lower jaw. Another important characteristic is the presence of 3-5 striae radiating downward, that are present on the gill cover.



1. *Schematization of Sardina pilchardus*
(https://www.researchgate.net/publication/309152822_Isolation_and_characterization_of_anthropogenic_particles_in_three_planktivorous_fishes)

1.1.2. Distribution

The species is distributed between 15°-66° N and 23°-42° E (Sinovčić et al., 2008), in the upwelling system of the eastern coast of the Atlantic, from the North Sea to Senegal, including Sea of Marmara, Black Sea and the Mediterranean Sea (FAO, 2022) [Fig.2]. In particular, it is present mostly in the Western Mediterranean Sea, rather than in the eastern part. This is due to the fact that the sardine is an eurytherm species and prefers temperatures ranging from 10 ° C to 20 ° C (Bini, 1979-1996).

2. *Distribuiton of Sardina pilchardus (ICES Stock Annex)*

1.2. Biology and ecology

1.2.1. Biology

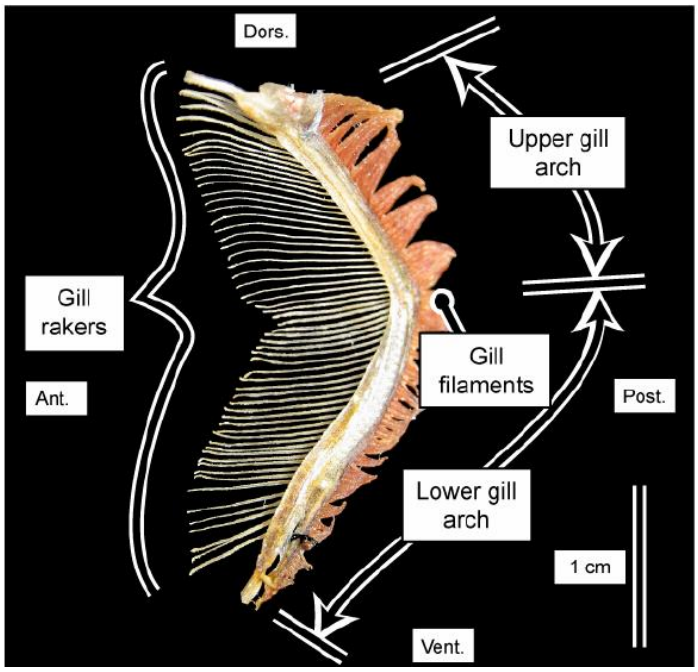
The sardine is a migratory, fast-growing and short-lived pelagic fish (Hure & Mustać, 2020). Although past studies showed that the sardine can reach 25cm in total length (TL), several recent studies have shown that the sardine can reach 20cm in areas such as Morocco (Keznine et al., 2020), while in the Adriatic they reach up to 17cm (Hure & Mustać, 2020). This is because in the Mediterranean, the sardine has a fast growth but reaches a shorter maximum length than in the Atlantic (Tsikliras & Koutrakis, 2013). The sardine, in Adriatic, spawns during the winter months, from October to April (Sinovčić, 1983, 1983–84), while the resting period extends from June to August, shown by the minimum weight of the gonads in this period (Mužinić, 1954). For this reason, they carry out two migrations during the year: one offshore in autumn/winter for reproduction and one inshore after the end of the reproductive period, in spring, to feed (Mužinić, 1973). This is because, during the reproductive period, these small pelagic fish need to find areas in which the abiotic conditions are best for the survival of the eggs and larvae (Škrivanić, 1973). Moreover, the sardine, as well as for the other clupeids, is a batch spawner (Roy et al., 1989). It has an asynchronous ovary, which has groups of oocytes at different stages of development. It is a species no

parental care, with external fertilization (Mužinić, 1954; Sinovčić, 1983-84, 1984) and the age of the first maturity is at the first years of age (Sinovčić, 1984; Sinovčić et al., 2003).

As regards the phenomenon of gregarism, the sardine forms schools only in the post-larvae stage and subsequent stages, before, the larvae are dispersed. Gregarism can be multispecies and take place with individuals of other species, but which have similar dimensions; this is known as “gregarism per size” (Donato et al., 2017).

1.2.2. Ecology

With other small pelagic fish as anchovy *Engralius encrasicolus* (Linnaeus, 1758), and round sardinella *Sardinella aurita* (Valenciennes, 1847), the European sardine has a key role, defined "waspe-waist" in ecology (Rice 1995, Bakun 1996), because transfers resources from to the lower levels of the food webs to the higher ones (Cury et al. 2000). In fact, its diet consists in plankton. In particular, the larvae are a selective zooplanktivorous, they mostly eat copepod eggs and nauplii, while the juveniles and adults (over 100-110mm in length) can use two modes of feeding; filters-feeding or particulate feeding, depending on feeding condition (James, 1986); so, they can eat both zooplankton and phytoplankton, in different proportion according to the area. For example, in the Adriatic Sea, the sardine are mostly zooplanktivorous and eat mainly copepods and decapod larvae (Hure & Mustać, 2020). The change in feeding between larvae and adults is due to the development of the filtering apparatus, which are the gill-rakers (Garrido & van der Lingen 2014). The filtering apparatus is composed of five pairs of branchial arches and, each of them, is composed of one series of gill-rakers [Fig.3] (Andreu, 1969), that appear already at 20-25mm in length (Costalago, 2012).



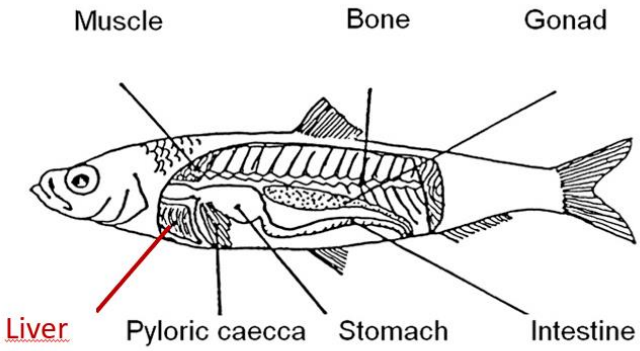
3. *Examples of gill-rakers in a cupleid fish (Rykaczewski, 2009)*

As sardines have high feeding rates, especially during the larval stages (Santos et al. 2006,), they carry out nictemeral migrations: during the day they move upwards, coinciding with the microzooplankton and mesozooplankton to pray on it (Andreu, 1969; Vučetić, 1963), while during the night they move into deeper waters to escape predators (Zwolinski et al., 2007).

1.3. The important role of the liver

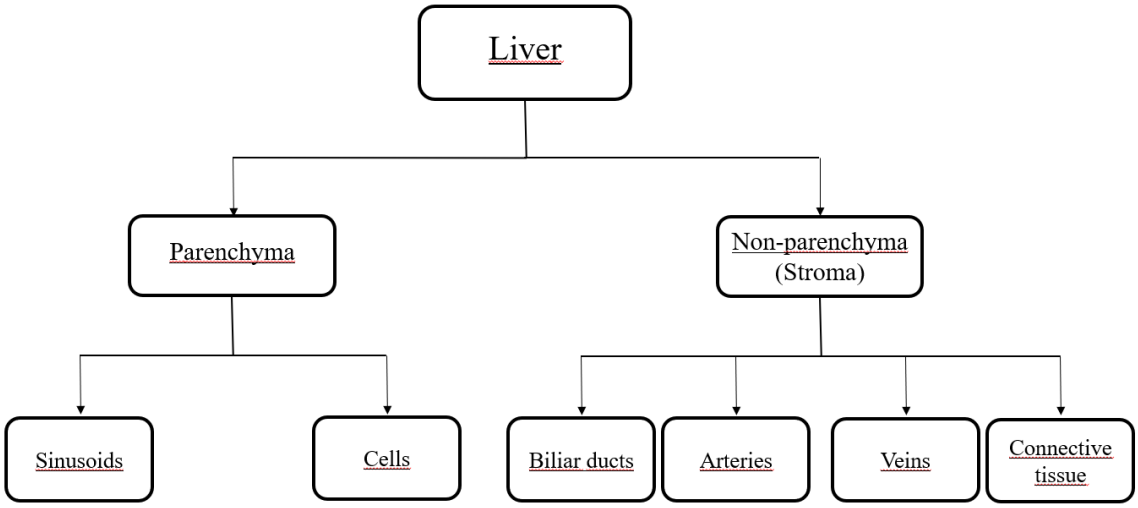
1.3.1. Liver morphology

The teleost liver is present in the cranial area of the general cavity [Fig.4], where all the organs are present. Some Teleost have a tri-lobed liver, while others show no lobulation (Bruslé & Anadon, 1996). Its size and volume depend by the disposition of the other organs (Bertolucci et al., 2008).



4. *Anatomy of Sardina pilchardus (image based on Carvalo, 2011)*

The liver is divided into two compartments: parenchyma and non-parenchyma (stroma) [Fig.5]. The parenchyma is constituted by cells (hepatocytes, macrophages, endothelium cells, biliary epithelial, Ito) and sinusoids; while the stroma presents: bile ducts, arteries, veins and connective tissue (Hinton & Lauren, 1990).



5. *Liver scheme in Teleost*

The liver is a highly perfused tissue, it receives blood from both the hepatic artery and the portal vein (Damjanov, 1996), which branch and convey the blood to the sinusoids.

The most part of the liver is constituted by hepatocytes, which are distributed in tubules or cordon (Hampton et al., 1985; Shore & Jones, 1989). Hepatocytes are polygonal cells, more precisely they are hexagons (Geyer, 1989).

Other type of cells are Ito cells, which have lipid drops with vitamin A, Kupffer cells, which are characterized by a phagocytic action and macrophages, in particular, melanomacropaghes (MM) (Munshi & Dutta, 1996).

1.3.2. The role of the liver in metabolism

The liver is a key organ because it performs various functions. It plays an important role in the metabolism, both in anabolism (synthesis of new substances) and in catabolism (degradation of more complex molecules into simpler molecules, with the release of energy) (Bruslé & Anadon, 1996). It receives the nutrients absorbed from the intestine via the portal vein, which carries 70-80% of the blood to the liver (Akiyoshi & Inoue, 2004; Rappaport, 1963); only the complex lipids, as the chylomitrones, are transported by lymph vessels (Rappaport, 1963).

Fish use mainly lipids and proteins as font of energy, poorly the carbohydrates (Cowey & Sargent, 1972; Walton & Cowey, 1979).

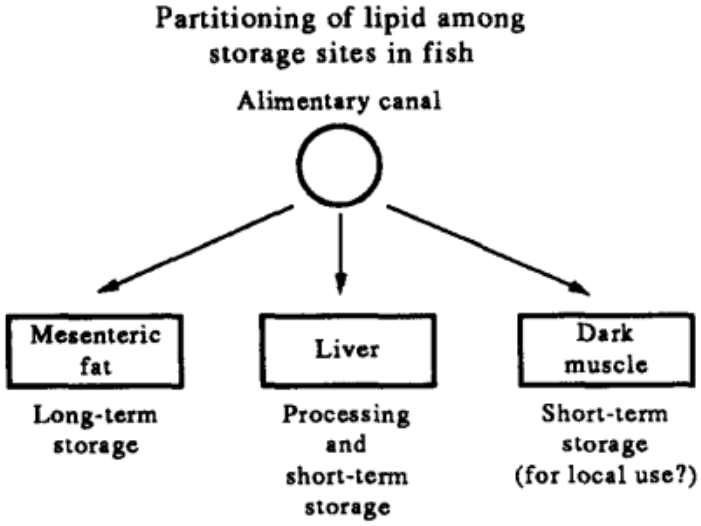
The muscle, which is the main mass of the fish, use glycogen to maintain the contraction activity; this glycogen comes from glucose which is produced via the gluconeogenesis process from amino acids, triacylglycerol and lactate (from anaerobic glycolysis). The main resource of gluconeogenesis is lactate (Moon et al., 1985), in fact, in a study on rainbow trout, Mommsen and Suarez (1984) have shown that the main role of gluconeogenesis is to convert lactate, produced by the muscle contraction action, in glucose. But, in other species, it has been demonstrated that the main resource was amino acids (Cornish & Moon, 1985). This shows how the main starting substrate is

species specific. Liver is the main organ in which gluconeogenesis occurs (Moon et al., 1985).

Other functions of the liver are the erythrocyte production, bile production and immune response (stimulates the action of granulocytes and lymphocytes).

1.3.3. The role of the liver in reproduction

Another important role of the liver is the lipid processing: it synthesizes new lipids starting from lipid received by the intestine. Furthermore, since lipids are insoluble molecules, the liver also secretes the lipoproteins that are used for their transport (*Very Low Density Lipoprotein, VLDL*) (Sheridan, 1988). In fish, differently from mammals, the storage and deposition of lipids is different. In fish, lipids can be stored in the liver, muscle or as mesenteric fat (Sheridan, 1994) [Fig. 6].

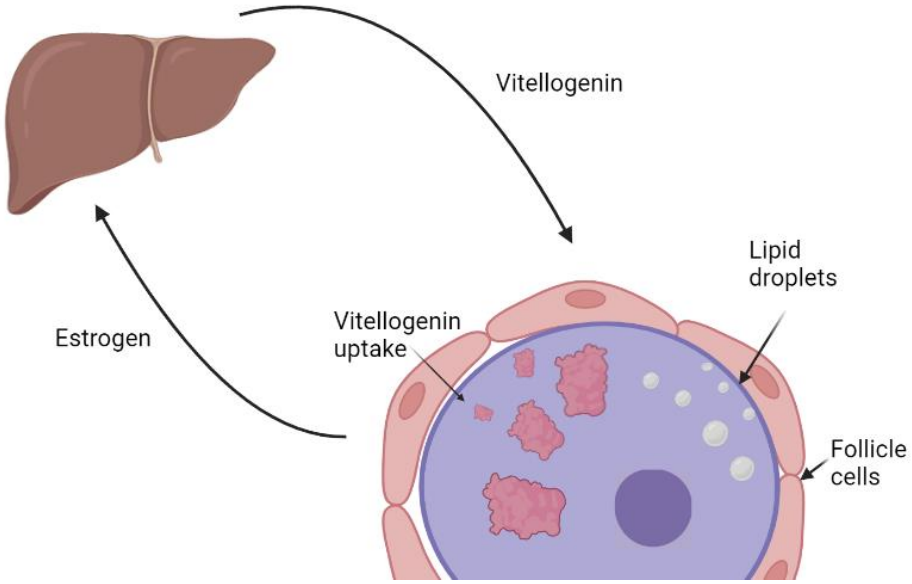


6. *Partitioning of lipid (Sheridan, 1994)*

The storage capacity of lipids can change according to the species, environmental factors and development of the organism (Henderson & Tocher, 1987; Sheridan, 1988, 1989). In the last case is important the interaction between reproduction and growth due to both needs of high level

of energy. In fact, lipids, in particular fatty acids, are the main energy resource of fish, not only for growth but also for reproduction, especially in females (Tocher et al., 1984a, b). In maturing rainbow trout females, it was observed that the free fatty acids and triacylglycerols levels in plasma and liver were higher than in immature females, suggesting the mobilization of the lipids, in the form of lipoproteins, from liver to ovary through the blood (Takashima et al., 1971, 1972).

Another important molecule produced by the liver for reproduction is vitellogenin. Vitellogenin is a glycolipophosphoprotein and its formation process is called "vitellogenesis". During this process, the ovarian follicles release large amounts of estrogen, which bind to receptors in the liver, resulting in the transcription and subsequent translation and maturation of vitellogenin (Hara et al., 2016) [Fig.7].

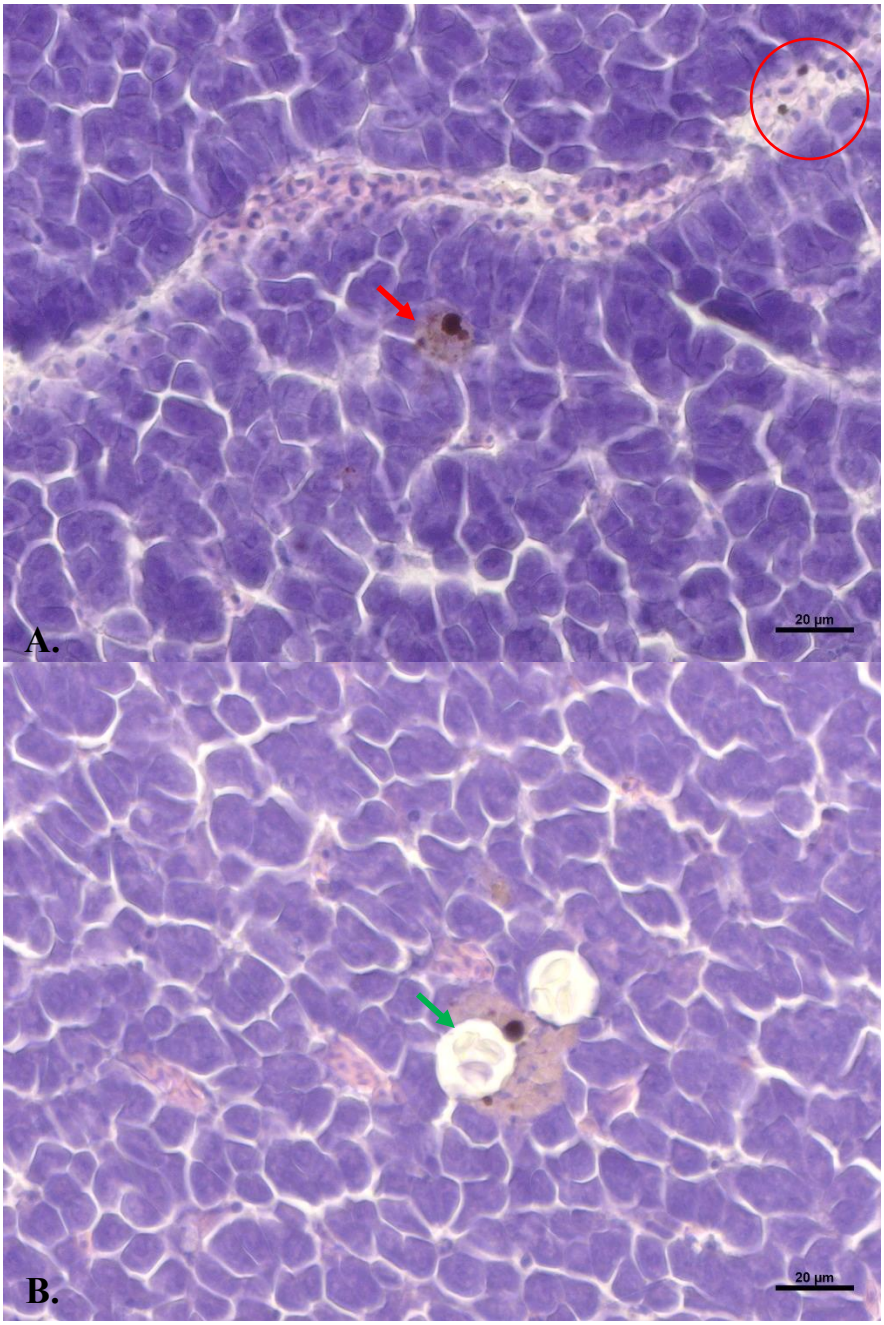


7. Vitellogenesis scheme

1.3.4. The role of the liver in immune defence

Finally, the last function that the liver can perform is the detoxification, determining the removal of possible toxic substances, which can be both endogenous (phagocytosis of exhausted cells, i.e. erythrocytes) and exogenous (i.e. heavy metals). To carry out this action it uses melanomacrophages (MM). Melanomacrophages can be interesting to study because, as there are not species-specific reagents for no-model organisms, they can be used as biomarkers for the immune response (Steinel & Bolnick, 2017). In addition to having an immune action, melanomacrophages also have a physiological action, as the phagocytosis of exhausted cells (i.e. erythrocytes) (Wolke, 1992).

Melanomacrophages are common in fish (Agius, 1980) as in other poikilotherms (Steinel & Bolnick, 2017). They can be present both in single form (MM) and in aggregate form (Melanomacrophages center, MMCs) (Agius 1980) [Fig.8] and the organs in which they are most found are the liver, spleen and kidneys, occasionally they can also be found in other organs (Agius & Roberts, 2003).



8. *Micrography of European sardine liver. [A] MMC indicated with red arrow and MMs in a blood vessels. [B] phagocytosis of a parasite by MMC (indicated with green arrow)*

Melanomacrophages can have variable shapes, sizes and pigmentations based on various factors such as: species, environmental conditions, stress, age, conditions of the individual (Fishelson 2006; Thorsen, Høyheim & Koppang 2006; Valavanidis et al. 2006; Weisman & Miller 2006). First of all, single

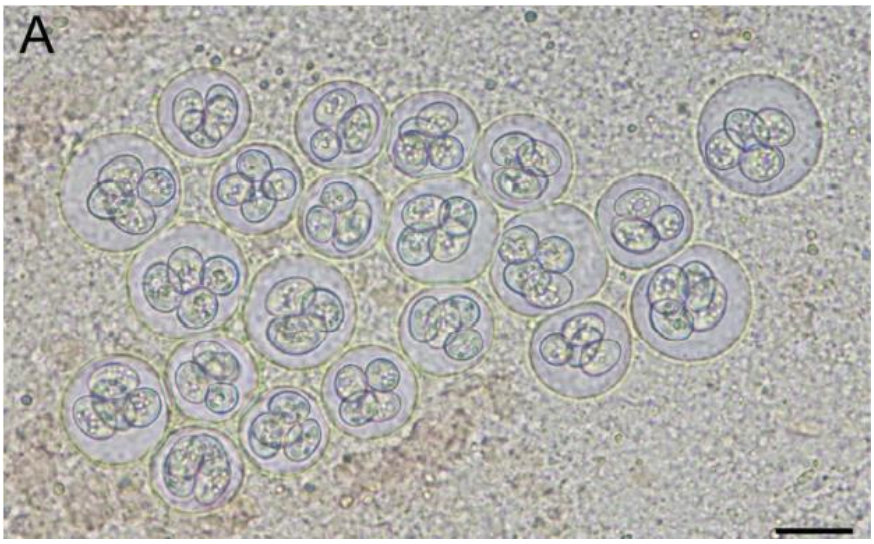
MMs travel freely in the blood and, due to diapedesis and migration phenomena, move within the tissue (Passantino et al., 2014). These, then, can form aggregates, the MMCs, to carry out their phagocytosis action. Within them the pigments can be melanin, lipofuscin or hemosiderin (Agius & Roberts, 2003). It has been shown how, on the basis of the action carried out by melanomacrophages, their content varies. For example, it has been found that in melanomacrophages which deal with the degradation of erythrocytes and which, therefore, accumulate iron, have hemosiderin (Agius & Roberts, 2003). On the other hand, beta-oxidation of fatty acids was found in melanomacrophages that accumulate lipofuscin, which is a secondary metabolite of beta-oxidation (Agius, 1985).

1.4. Coccidiosis

Coccidiosis is caused by protozoan parasites and causes gastrointestinal infections (Britannica, 2022). Coccidian parasites include different types of species (*Toxoplasma, Eimeria, Hammondia, Isospora, Neospora*), belonging to the Apicomplexa. These species are distinguished by certain characteristics that they exhibit during asexual reproduction (sporozoites and merozoites) (Mai et al., 2009).

Coccidia are obligate parasites and are usually specific to a host and also to an organ, especially the gut. Nevertheless, they can lead to extra-intestinal infections, especially in fish (Davies & Ball, 1993). Their life cycle consists of an asexual and a sexual phase. In terrestrial vertebrates, resistance cysts, oocysts, are released into the environment via the intestine and remain there until they find another host (Lindsay & Todd, 1993). In fish, oocysts can already sporulate inside the host and in some cases an intermediate host is required (Molnàr et al., 2012).

Fish can mostly be parasitized by three genera; *Goussia* (Labbè, 1896), *Eimeria* (Schneider, 1875) and *Calyptospora* (Overstreet, Hawkins & Fournie, 1984) (Xavier et al., 2021). Almost all coccidia have oocysts with four sporocysts each and two sporozoites in each sporocyst (Lom & Dyková,1992; Davies & Ball, 1993) [Fig.9].



9. *Example of morphotype of Goussia clupearum in homogenize liver of Atlantic herring (Friend et al., 2016)*

Although fish are commonly prone to coccidia infections, few information are available about them (Friend et al., 2016). What is known about it is that they cause a worsening of physical conditions and that they cause infections in wild populations, associated with necrosis and sloughing of intestinal cells (Abollo et al. 2001, Lovy & Friend 2015).

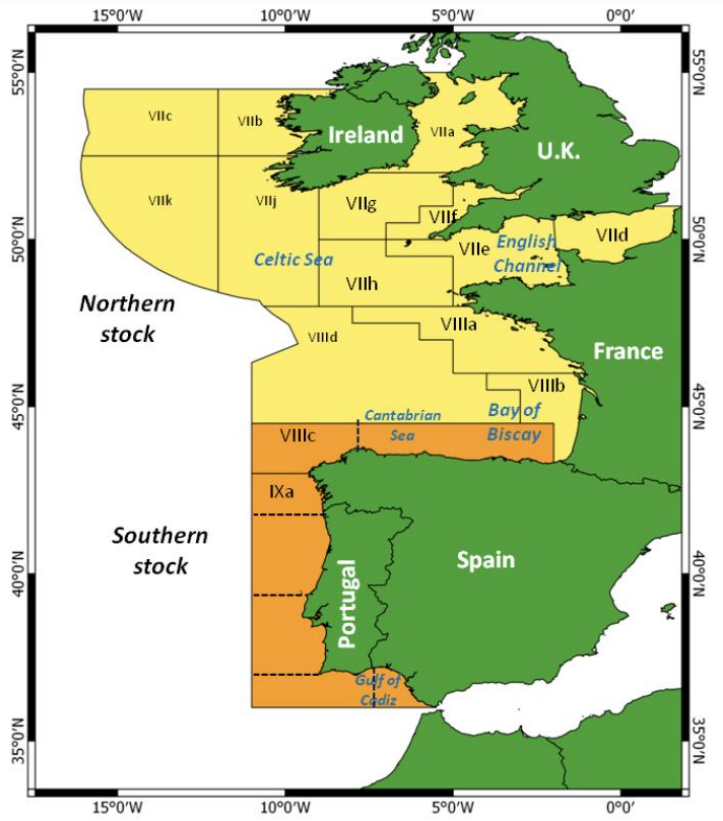
1.5. Fishing effort

1.5.1. Fishing in the Atlantic

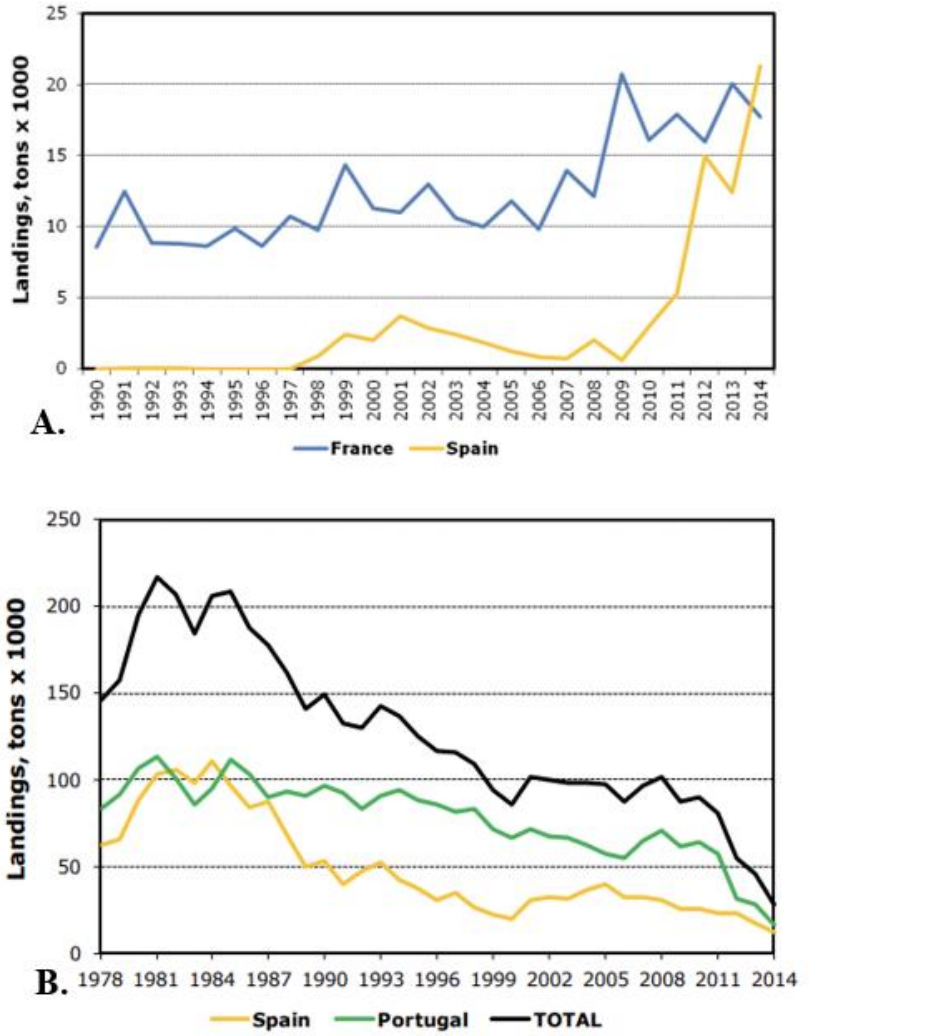
European sardine fishing is very important in several countries, such as France, Spain and Portugal in the Atlantic Ocean (Silva et al., 2015), while in the Mediterranean sardine fishing is especially important in the Adriatic Sea (Santojanni et al., 2005).

Sardine fishing is carried out using two types of boats: purse seine and pelagic trawl nets (*volante*). In particular, purse seines are mainly used in countries such as France, Spain and Portugal (98% landed) (Silva et al., 2015), while in the Adriatic there is a greater use of pelagic trawl nets (Santojanni et al., 2005).

The Atlantic sardine stock is divided into two parts: the northern stock (France, Spain, UK, Netherland) and the southern stock (Spain and Portugal) [Fig.10]. While the northern stock from 1990 to 2014 recorded an increase in landed, the southern stock from 1978 to 2014 recorded a decline (Silva et. al., 2015) [Fig.11].



10. *Division of the North Atlantic Sardine Stock (Silva et al., 2015)*



Source: ICES, 2015.
Note: In Spain, catch regulations started in 1985. In Portugal, catches were regulated from 2000 to 2004 and again since 2010.

11. *(A) Northern stock: sardine landings by country in ICES Subarea VII from 1990 to 2014. (B) Northern stock: sardine landings by country in ICES Subarea VIII from 1990 to 2014. (C) Stock: total landings and by country between 1978 and 2014 (Silva et al., 2015)*

This decline is due to overfishing of the sardine stock. In fact, in 2012, after the drastic decrease recorded in 2011 (72%), restrictions were established in Portugal. Despite this, there has been a decline in biomass, especially in Spain (Silva et al., 2015). Another reason for the decline in biomass is recruitment. In fact, since the 1990s, it has been noted that every low recruitment

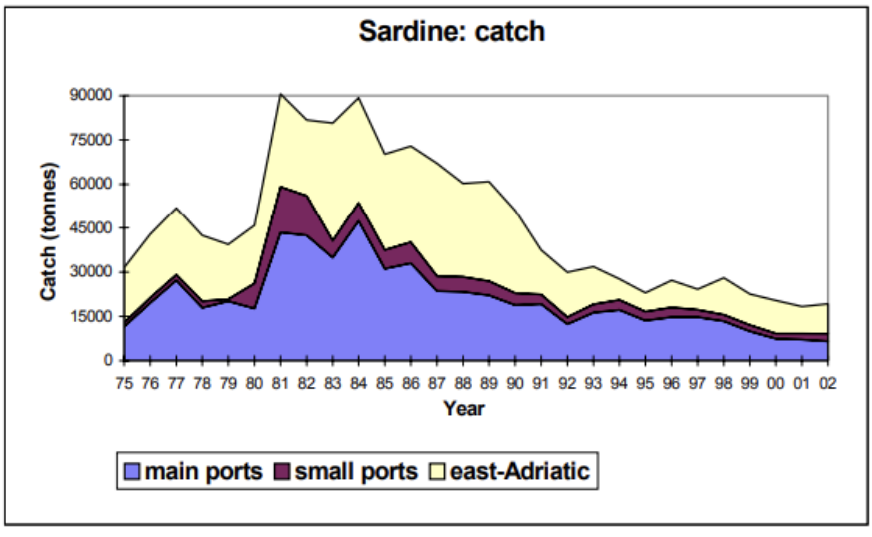
corresponds to a low biomass, after a couple of years. Indeed, the lowest biomass levels were recorded in 2015 (139,000 tons), not only for overfishing, but also for the nine years of low recruitment. Unfortunately, the factors that determine this low recruitment are not specifically known (Silva et al., 2015) but it is known that recruitment is strongly linked to environmental factors, as temperature and hydrography (Houde, 2008).

Unlike the southern stock, the northern stock until 2015 was never regulated, except for the minimum size at 11cm, due to the fact that it was never needed (Silva et al., 2015).

1.5.2. Fishing in the Adriatic

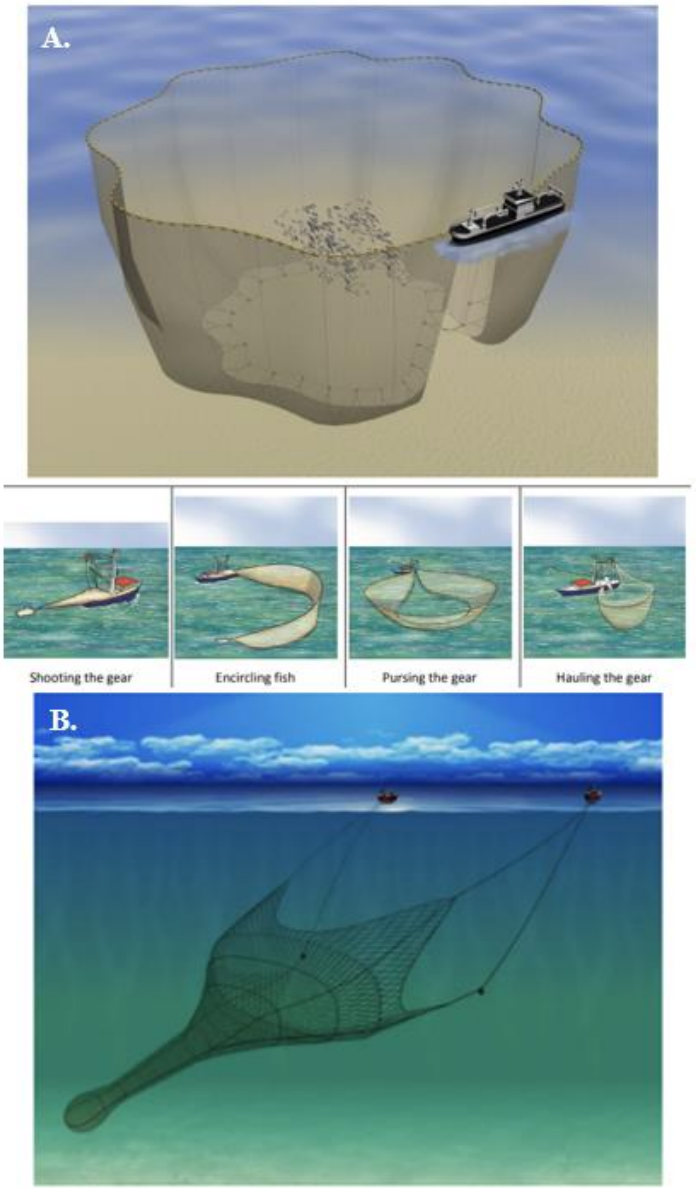
The European sardine is diffuse especially in the Northern and Central Adriatic (GSA17) respect to the Southern Adriatic (GSA18) (Santojanni et al. 2005). In the Adriatic, between 1970 and 2005, sardines and anchovies made up 41% of the total catch. In particular, in Italy, they represented, on average, the 35% of landed, of which sardines represent 16%. In Croatia, on the other hand, most of the landings concern sardines (Morello & Arneri, 2009).

In 1981 Italy reached its peak of sardine catches, landing 59,000 tons. In the following years, however, there was a decline in the stock due to the overexploitation of the resource. The same happened in Croatia, which reached the maximum of landings (40,044 tons) in 1983 and 1987 (38,439 tons), after which there was the collapse after 1990 (Cingolani et al., 2003).



12. *Sardine catches in Northern and Central Adriatic Sea (Cingolani et al., 2003)*

In the Adriatic, as well as in the Atlantic, two types of boats are used for fishing small pelagics: pelagic trawl nets and purse seines (Santojanni et al., 2005) [Fig.13]. In particular, in Croatia they are fished only by purse seines (204 vessels) (GFCM, 2014). Instead, in Italy, in 1959, purse seines have been largely replaced with the use of pelagic trawling nets (*volante*), which act in pairs. The purse seine is used above all in the south of Ancona, on calm nights (except for those of a full moon): the main boat attracts the fish to the surface thanks to a light source, after which, with the help of another boat, surrounds the school of fish with a net; this type of purse seine is called *lampara* (Morello & Arneri, 2009). It is used in the season from April/May to November (Morello &Arneri, 2009), while during the winter it is not used or is replaced by the use of pelagic fishing nets (Santojanni et al., 2005). The Italian fleet is made up of 55 pairs of pelagic trawling nets and 40 lampare.

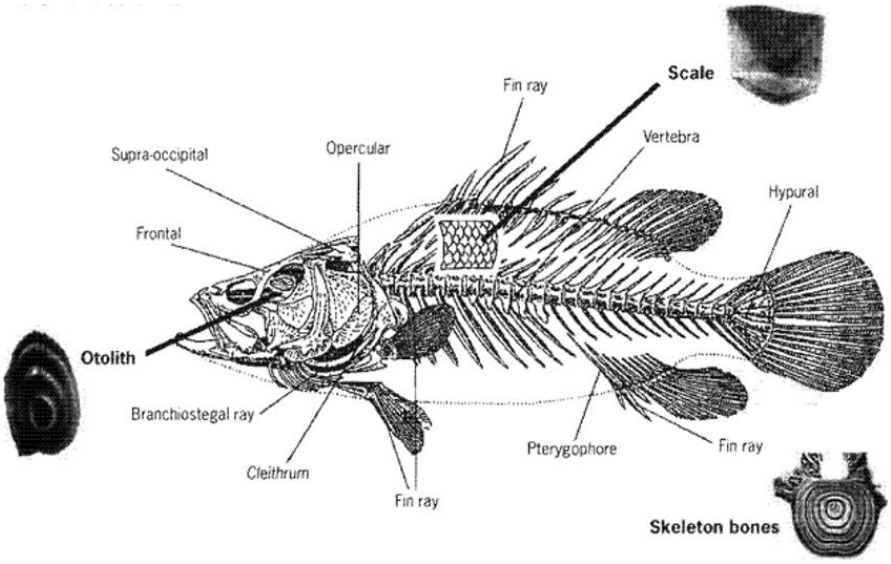


13. *(A) Purse seine and (B) pelagic pairs trawling schemes (Weissemberger, 2015)*

1.6. Age and growth

1.6.1. Age determination structures

The first age determinations, on an annual basis, were made using rings in the vertebrae of eel, in 1759 (Hederstrom, 1959). Later on, in 1888, scales were used (Carlender, 1987). The analysis of otoliths was first done by Reibisch in 1899 in *Pleuronectes platessa* (Ricker, 1975) [Fig.14].



14. *Different age determination structures (Panfili et al., 2002)*

However, this annual analysis is not suitable for determining the age of juveniles that have not reached a year yet. In fact, subsequently, other analysis were made on the daily deposition (Pannella, 1974; Struhsaker & Uchiyama, 1976; Brothers et al., 1981). Pannella, in 1971, observed that, in fish of temperate seas, between one annual ring and another, there are about 360 finer rings.

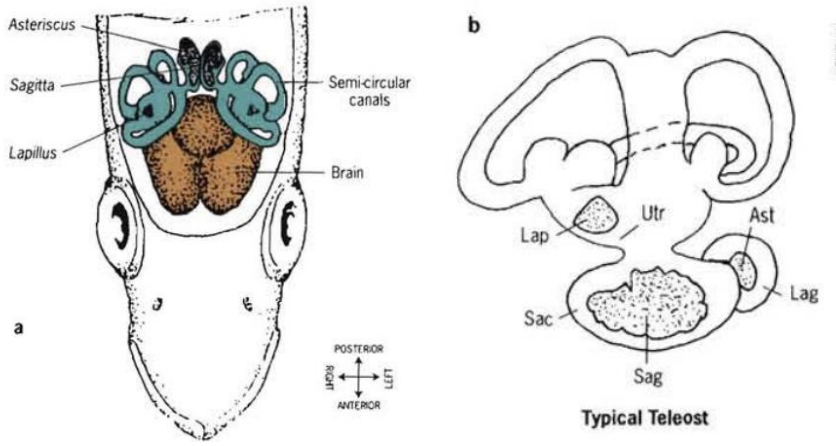
The analysis of otoliths is fundamental for determining the age and growth. Indeed, informations on age in the early stages of life can help us understanding the effects of environmental change on growth. While studies of this type on adults are used to determine the fishing effects of the stock (Jones, 1992).

As mentioned above, there are three types of structures from which age can be measured: bones, scales and otoliths. The most used are the otoliths, which allow to observe both daily and annual patterns. Scales, even if the organism does not necessarily have to be killed to be able to take them, can't be used for reading the daily growth, as well as the bones. Furthermore, unlike bones and otoliths, scales can't be completely reliable because they can be lost and replaced and, at a certain age, they stop growing (Jones, 1992).

1.6.2. Otoliths

There are three types of otoliths in fish: *lapilli*, *sagittae* and *asteriscus*. *Sagittae*, the largest otoliths, are the most used in reading the age. Sometimes *lapilli* are also used, while *asteriscus* are not legible (Jones, 1992).

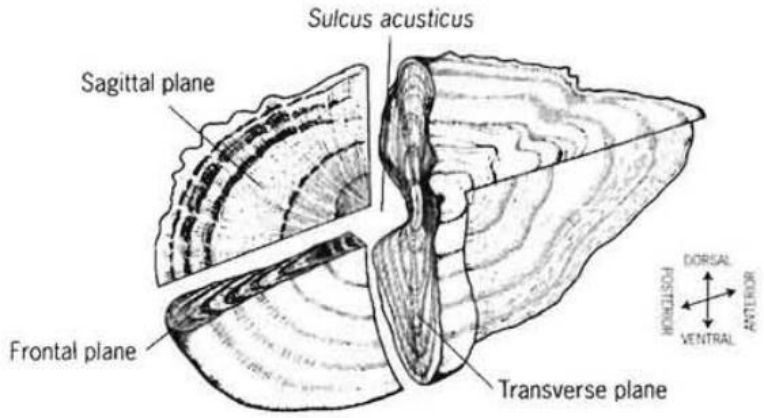
These three types of otoliths are located in the inner ear, which has both an acoustic and static function. The inner ear is made up of several canals and sacs, which contain the endolymph. In Teleost there are three semicircular canals, arranged orthogonally to each other. These canals flow into three sacs called *utriculo*, *sacculo* and *lagena* which contain, respectively, *lapilli*, *sagittae* and *asteriscus* [Fig.15]. These otoliths have a mechanoreceptive function and stimulate the kinocilia of the *macula* (sensory epithelium). The kinocilia transform the mechanical signal into a nerve impulse which is transmitted to the brain (Panfili et al., 2002).



15. *Location of otoliths in the inner ear of Teleost. (A) Frontal section of the cranium of a teleost and dorsal view of the vestibular apparatus. (B) Otoliths within the Teleost labyrinth system.*

Otoliths have a different shape from species to species (Panfili et al., 2002) and this difference may result due to both genetic and environmental variations (Lomberte & Lleonart, 1993; Campana et al., 1995; Torres et al., 2000). Thanks to this inter-specific variety, otoliths are used as taxonomic characters (Hecht, 1979).

Otoliths have three orientation planes: transverse, sagittal and frontal [Fig.16]. It is important to define the orientation plane when a section of the otolith is carried out.



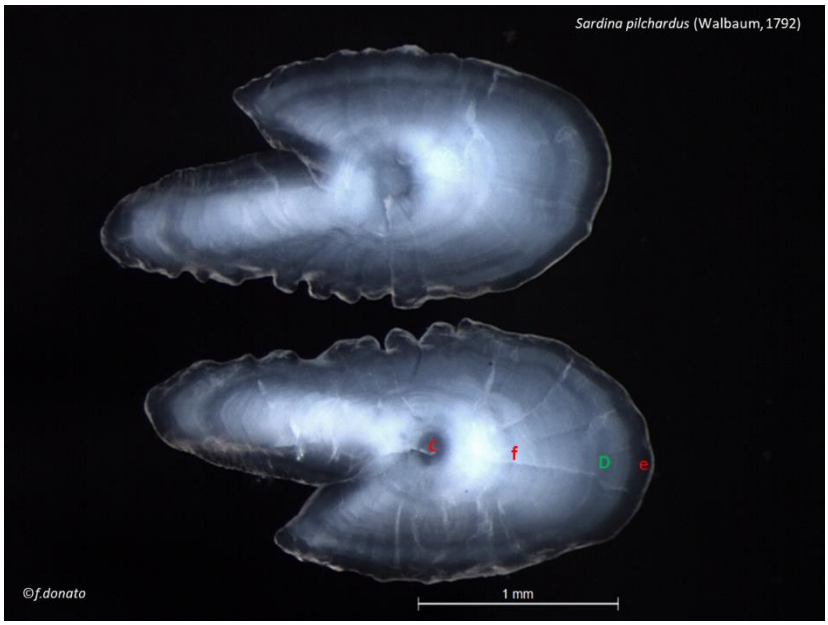
16. *Different orientation planes of the otolith (Panfili et al., 2002)*

Sagitta develops mainly along the anterior-posterior axis. It has a rostrum in the front and an anti-rostrum on the back (Panfili et al., 2002). The ventral part is characterized by the presence of the *sulcus acusticus*, a groove that is in direct contact with the *macula* of the *sacculo* (Dunkelberger et al., 1980; Fay, 1984; Platt & Popper, 1981).

1.6.3. Analysis of otoliths

As previously mentioned, otoliths have both daily and annual growth. The annual growth is determined on the basis of the seasonal one, which is characterized by the alternating deposition of a light band, L-zone, rich in calcium carbonate and a dark band, D-zone, rich in a protein matrix (otoline). Together, these two bands form the annulus (Panfili et al., 2002).

These bands are around the core of the otolith, called *primordium*. *Primordium*, which forms during the latter part of the egg stage in fish, can have a circular, elongated or multiple shape depending on the species (Panfili et al., 2002) [Fig.17].



17. *Representation of a clupeid sagittae. C= core or nucleus; f= false ring or check; green dots= winter ring; e=hyaline edge; D= D-zone*

2. AIM

Sardina pilchardus (Walbaum, 1792) is an important resource both from an ecological and an economic point of view. From an ecological point of view, it plays an important role within the food network, being involved in both up-regulation and down-regulation processes. While from an economic point of view it represents one of the most important food resources, both in the Atlantic and in the Mediterranean Sea, in particular in the Adriatic Sea.

Environmental conditions and hydrogeographic factors strongly determine the abundance of this small pelagic fish, making it a possible indicator of environmental variations and indeed, important to study.

In fact, the objective of this work, carried out over 12 months, was to determine the state of health of the central Adriatic Sea stock of sardine. To this porpoise, histopathological analysis of the liver was performed, investigating the presence of single and centres of melanomacrophages (MMs and MMCs) and other histopathological parameters such as: presence of hemolysis, infiltration of white blood cells, presence of parasites, vacuolation and necrosis of the tissue. All these parameters were then related to the seasonality, to size (length and weight), to age (analysis of otoliths) and to fishing effort (landings data).

This was possible thanks to the collaboration between the Polytechnic University of Marche, CNR-IRBIM Ancona and the University of Catania.

3. MATERIAL AND METHOD

3.1. Sampling

The samples were taken from the Ancona *volanti* fleet, within the area between Ancona and Pesaro (Adriatic Sea. GSA 17. FAO Geographical Area 37, Subarea 37.2).

The sampling period started in April 2021 and ended in March 2022. The sampling took place every month, except for the months of June and October due to the fishing stop.

The sample, a 1-3 kg box [Fig.18A], was taken each month when the boats returned to port and sampled the following day. Biometrics (weight and length) and sex of approximately 100 individuals, were monitored each month, for a total of 1032 individuals. The length of each individual was measured from the tip of the snout to the tip of the caudal fin, to the nearest millimeter [Fig.18B], and weighted to the nearest gram. In addition those specimens, a representative sub-sample of each size class (setting the size class at 0.5 cm) composed by 3 males and 3 females, was sampled each month for histological and otoliths analyses. A total of 321 animals were sampled, distributed into 10 size class (from 11 cm to 15.9 cm).

In particular from those individual, the whole liver was sampled, weighed (0.001g) and fixed in formol (formaldehyde and glutaraldehyde) [Fig.18C].

After that, the eviscerated samples were stored in a cold room at -20 ° C until they were transported to the CNR for the extraction of the otoliths.

18. *(A) Box of sardine. (B) Example of sardine sampling. (C) Liver of sardine*

3.2. Indices

Hepato-Somatic Index (HSI)

The hepato-somatic index (or HSI) is a ratio between the weight of the liver and the weight of the uneviscerated organism according to the following formula:

$$HIS = W_H / W_T * 100$$

Where W_H is the weight of the liver and W_T is the total weight of the sardine.

Fulton's Condition Factor (K)

The condition factor (K) is a sensitive measure of change of body condition. It is calculated as a ratio between the weight and the length, using this formula:

$$K = (W / L^3) * 100$$

3.3. Histological analysis

Inclusion

To perform the histological analysis the samples were embedded in paraffin. Since only female samples are analysed, the total number of samples included is 161.

First of all the sample have been prepared for inclusion: it has been subject to 2 rinses of 15' in ET-OH 70% to eliminate the excess fixative. Then, the sample was put inside a biocassette which was then immersed in a growing series of ethanols (starting from ET-OH 70%), followed by passages in xylene and, finally, in paraffin. The passages in ethanol have been used to dehydrate the sample, while those in xylene have been used to eliminate the alcohol and prepare the sample for passage in paraffin. The paraffin in which the samples have been placed last is the blue paraffin, less soluble and penetrating than the white paraffin but which has a higher melting point which makes it easier to process in the presence of high temperatures.

The various steps were carried out according to the following protocol:

- 45’ ET-OH 70% (or overnight)
- 45’ ET-OH 80%
- 45’ ET-OH 95%
- 1h ET-OH 100% (I)

- 1h ET-OH 100% (II)
- 15’ Xylene (I)
- 30’ Xylene (II)
- 2h white paraffin (46-48°C)
- 1.30h blue paraffin (56-58°C)

At the end of this process there was embedding in blue paraffin. After that the sample has been left to solidify for at least one night.

Microtome

Following the inclusion, the sample was cut with the microtome, to a thickness of 4-5 μm. Each section was then placed in lukewarm deionized water to facilitate its distension and, subsequently, was placed on a slide. Three sections were made for each slide, cut at a distance of 10-15 cuts from each other.

The slides were then left to dry at room temperature for at least one night.

Staining

Finally, the last step in the histological process is staining. The sample was stained with Hematoxylin & Eosin according to the following protocol:

- 10’ Xylene dewaxing (I)
- 10’ Xylene dewaxing (II)

- 5’ ET-OH 100% (I)
- 5’ ET-OH 100% (II)
- 5’ ET-OH 95%
- 5’ ET-OH 80%
- 5’ ET-OH 75%
- 10’ Current tap water
- 1.30’ Hematoxylin
- 5’ Current tap water
- 40’’ Eosin
- 2’ Current tap water
- Fast steps in growing ethanol (70%, 80%, 95%, 100% (I))
- 1’ 100% (II)
- 15’ Mounting xylene

The first steps in xylene were used to deparaffinized the sample. Subsequently, the decreasing passages in ethanol served to hydrate the sample, while the passages in running water served to remove the excess ethanol and dye. Finally, the steps in increasing ethanol dehydrated the sample. The last step in mounting xylene was used to clean the sample from any paraffin residues and to facilitate mounting of the coverslip.

Optical microscope

The stained sections were analysed under the ZEISS optical microscope (AXIO Imager M2) at different magnifications. Subsequently, two random photos were taken at 40x magnification for each section for a total of six photos per slide. These were then used for the quantification of melanomacrophages.

Instead, parasite oocysts were counted at 20x magnification. This occurred randomly for two replicates for each section, for a total of six replicates. The health of the liver was assessed at the same magnification.

ImageJ

ImageJ software was used for the quantification of melanomacrophages. The criteria for the analysis of melanomacrophages were the following:

- In case an MMs or an MMCs was partially present inside the photo it was not counted.
- Only when the edges of the capsule or the MMs cells were visible were they counted.
- In cases where the staining was very dark or two or more MMs cells were distinguishable, it was considered MMCs.

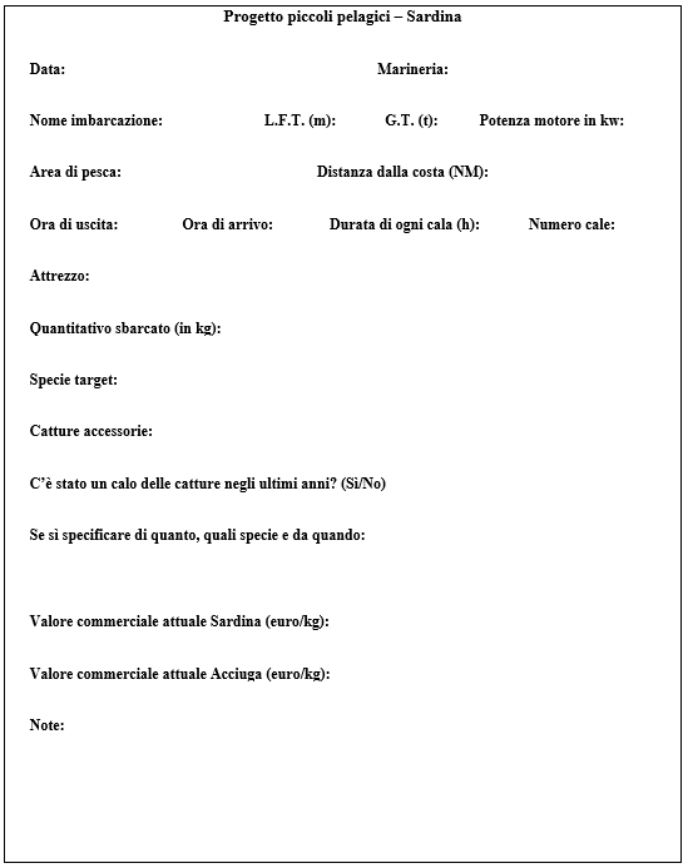
Subsequently, for each sample, an average was made of the area of MMs, MMCs and the number of MMs and MMCs present in each section. This area was then calculated as a percentage in relation to the area of the section.

3.4. Criteria for the classification of abnormalities of the hepatic parenchyma and blood vessel

During the histological analysis, abnormalities were found in both the hepatic parenchyma and blood vessels. The abnormalities were divided into 4 different categories for both hepatic parenchyma and blood vessels. These categories were then used to determine two types of grading, one for hepatic parenchyma and one for blood vessels, respectively. The grading goes from 0 to 4, where 0 means that there are no abnormalities and 4 that all of them are present.

3.5. Fishing effort analysis

Each month, at the time of recovery of the landing, a logbook was compiled for each boat (all the eight boats of Marineria di Ancona). In the figure [Fig.19] it is possible to see an example of the logbook.



Progetto piccoli pelagici – Sardina

Data: **Marineria:**

Nome imbarcazione: **L.F.T. (m):** **G.T. (t):** **Potenza motore in kw:**

Area di pesca: **Distanza dalla costa (NM):**

Ora di uscita: **Ora di arrivo:** **Durata di ogni cala (h):** **Numero cale:**

Attrezzo:

Quantitativo sbarcato (in kg):

Specie target:

Catture accessorie:

C'è stato un calo delle catture negli ultimi anni? (Si/No)

Se sì specificare di quanto, quali specie e da quando:

Valore commerciale attuale Sardina (euro/kg):

Valore commerciale attuale Acciuga (euro/kg):

Note:

***19**. Example of logbook*

From these data it was possible to determine the fishing effort, the mean commercial value and the fishing areas.

3.6. Otoliths analysis

Extraction

The extraction of otoliths of all sampled organisms was carried out at the CNR.

A sagittal cut was made from the base of the skull with a scalpel to extract the otoliths from the sample. Subsequently, with the help of tweezers, the skull cap was removed. This allowed us to have an integral view of the brain, which has been completely removed to extract the otoliths present at its base (Carbonara & Follesa, 2018).

Once extracted, the otoliths were cleaned of any residual organic matter, dried and inserted into the eppendorf. After 20-30 days it was possible to read them.

Age reading

Otoliths age reading was carried out under reflected light using a stereomicroscope at 10-16x magnifications linked to a digitized computer video system (Leica Application Suite 4.3.0.) through a CCD videocamera (Leica DFC 420).

Individual age was estimated by counting the hyaline (L-zone) and opaque (D-zone) bands, from the core to the otolith margin (edge), assuming they were laid down annually. Two blind readings were carried out by a single reader, and the mean value taken as individual age.

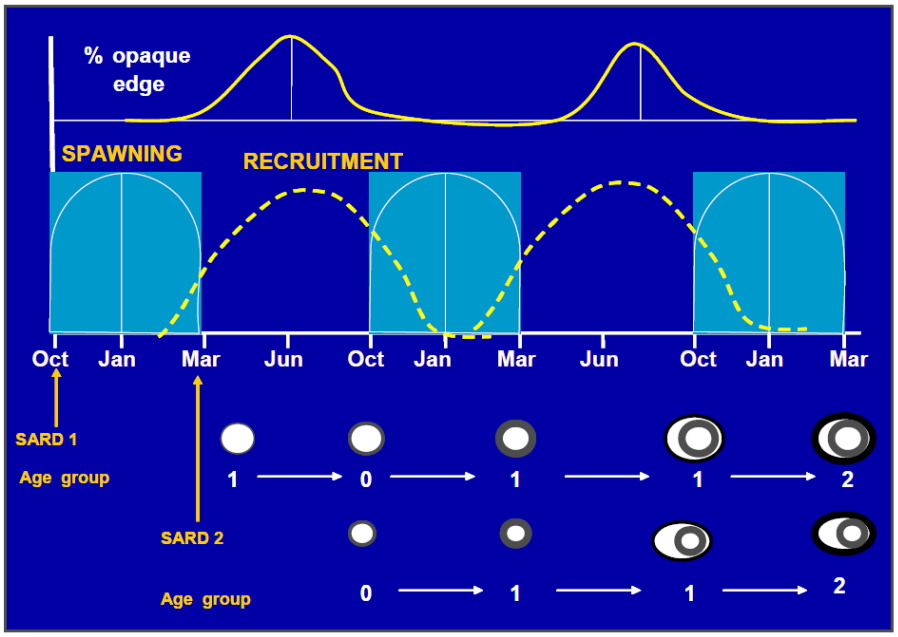
The criteria used for otolith age reading of the otolith are the following:

- The first of January is adopted as the birthdate (Williams and Bedford, 1974). Consequently, if an otolith is collected from a fish caught in the first semester of the year (winter/spring) the age group assignment will correspond to the number of hyaline zones present, including the edge if translucent. If the otolith is extracted from a fish caught in the second semester (summer/autumn) of the year the age group assigned will correspond to the hyaline zones completely formed, if the edge of the otolith is hyaline it will be not considered [Fig.20].
- The nucleus or core is hyaline because the spawning period occurs in winter.
- The distance measured from the core to the first true hyaline band (to about one millimeter) must be modified (reduced) by at least 10-15%, therefore only rings smaller than 0.85 mm should not be considered true rings.
- The extended spawning period (November to May) results in varying sizes of the first growth zones. Consequently, a large variation in the distance between the nucleus and the first hyaline ring can be expected. This must be kept in mind when interpreting the first hyaline and opaque areas. In fact, the rule says that the first hyaline ring should not

be considered as such, when its distance from the nucleus is proportionally less than the distance between this ring and the next.

- The code "99" was used to the otoliths too difficult to read or unreadable, often and without any hesitations. Considering the high number of read otoliths of the same size class, it is statistically preferable not to use the problematic otoliths in the calculation.

The ageing scheme for the species with birthday set on 1st January such as the sardine, is schematized in the following figure [Fig.20].



20. *scheme of the birthdate and otolith margin conventions for sardine age*

Data analysis

The relationship between weight and length was analyzed using the following formula:

$$W = aL^b$$

Where W is the weight of the individual, L the length and a and b two constants that vary according to the species. The value b depends on the type of growth of the individual and can take a value between 2 and 4.

To determine the accuracy of the age estimate between the two readings, two indices were calculated: the Average Percentage Error (APE) and the Coefficient of Variation (CV).

The APE was calculated using the formula:

$$APE_j = 100 \times \frac{1}{R} \sum_{i=1}^{R} \frac{|x_{ij} - x_j|}{X_j}$$

where R is the number of readings for each individual; xij is the i-th age attributed to the individual j-th; xj is the average value of the age of the individual j-th.

The mean APE was calculated using the formula:

$$APE_{mean} = 100 \times \sum_{j=1}^{n} APE_j$$

The CV was calculated with the formula:

$$CV_j = 100 \times \frac{\sqrt{\sum_{i=1}^{R} \frac{(x_{ij} - x_j)^2}{R-1}}}{X_j}$$

where Xij is the i-th age attributed to the individual j-th, Xj is the average value of the age of the j-th individual, R is the number of readings for each individual.

The mean CV was calculated with the formula:

$$CV_{medio} = 100 \times \sum_{j=1}^{n} CV_j$$

All these analyses were carried out with Excel software.

3.7. Statistical analysis

The statistical analysis was carried out using the GraphPad Prism8 software for the ANOVA and Pearson's correlation. Significance is set at a p-value <0.05. For the Pearson correlation the data were normalized. Furthermore, the months have been transformed into seasons using the following code:

Season	**Months**	**Code**
Spring	April May	1
Summer	July August September	2
Autumn	November December January	3
Winter	February March	4

4. RESULTS

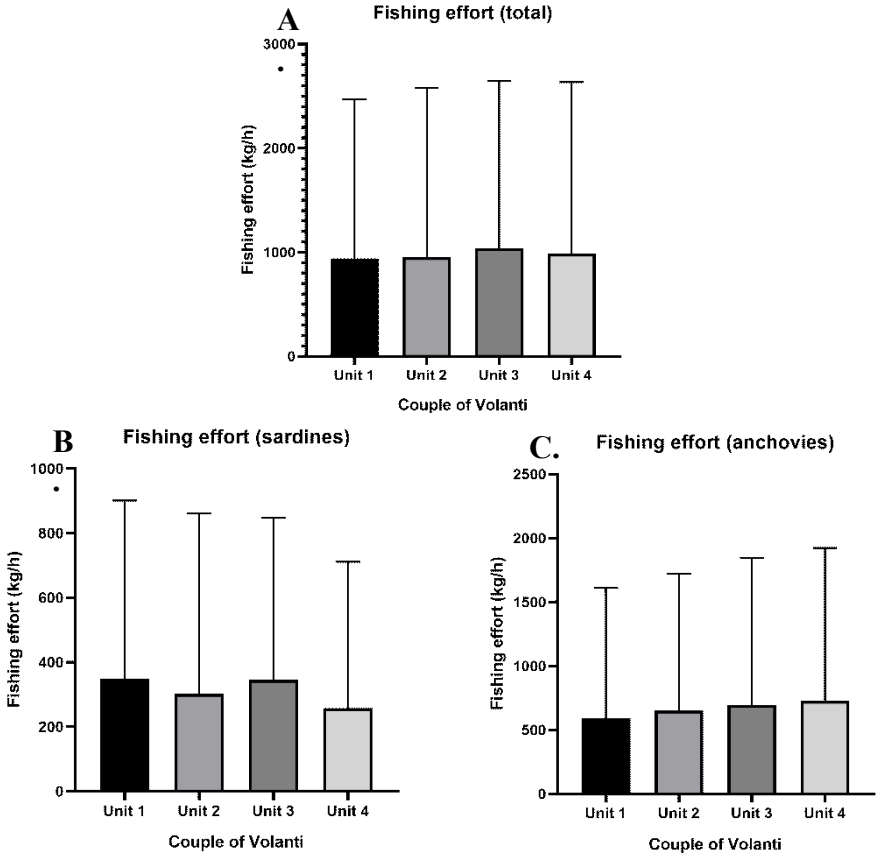
4.1. Fishing effort

As for the data relating to the Marineria di Ancona, the eight boats have an overall length (L.F.T.) between 24.95 and 28.6m, a gross tonnage (G.T.) between 93 and 141 tons and an engine power (kW motor) between 250 and 590 kW [Table 1]. These boats were considered as 4 fishing-units, each consisting of a pair of *Volanti*.

Vessel name	L.O.A. (m)	G.T. (t)	Motor kW
Vittorio Padre	24.95	101	408
Dearpa	27.85	114	588,2
Gigante	28.6	108	456
Mirage	25	101	250
Elnà	26.25	108	478
Benhur	25.75	132	521
Maretto	27.17	93	590
Labrador	27.35	141	333

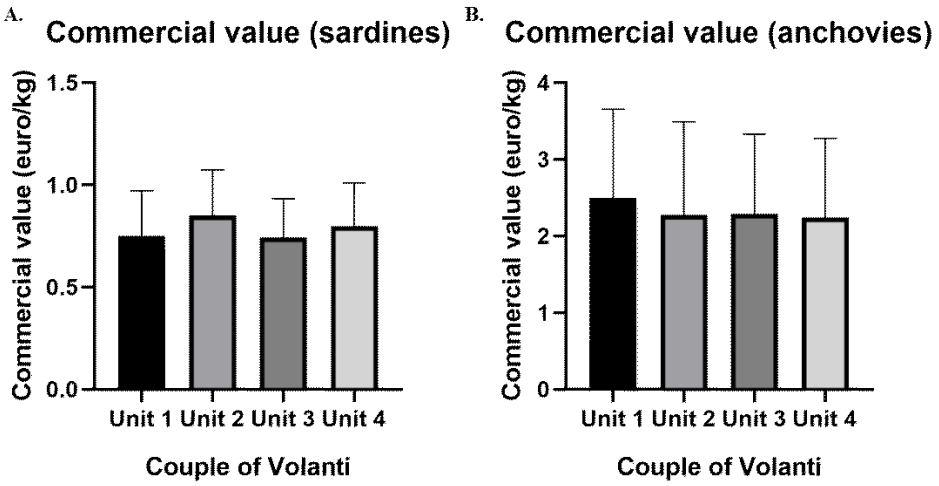
1. *Main features of Marineria di Ancona*

In the graphs representing the total fishing effort [Fig. 21A], for sardines [Fig. 21B] and anchovies [Fig. 21C] there is, in none of the three cases, a difference between the mean fishing efforts of the various units. However, it's possible to appreciate the high variability that characterizes each unit.



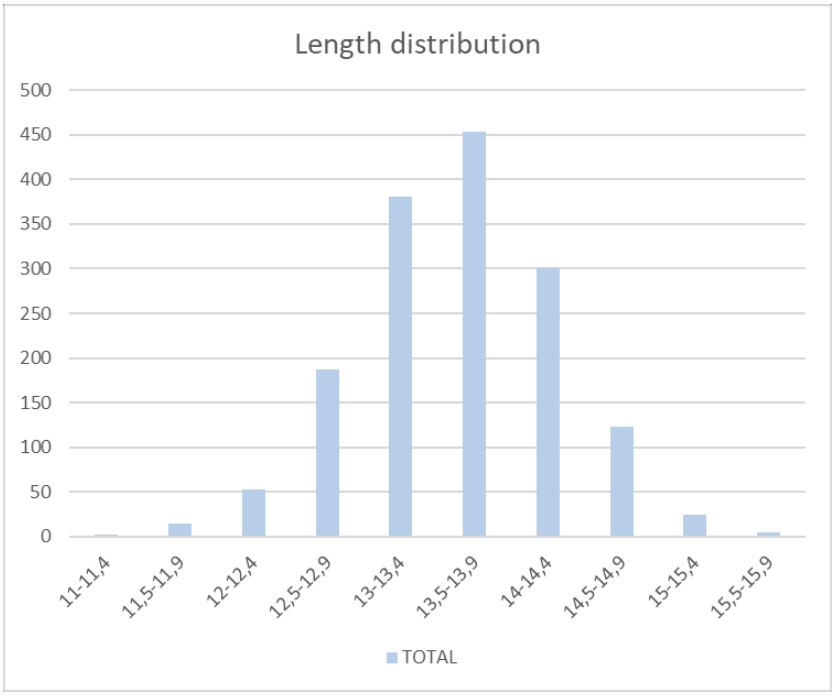
21. *Fishing effort. (A) Total fishing effort. (B) Sardine fishing effort. (C) Anchovies fishing effort*

On the other hand, as regards the commercial value, both for sardines Fig.22A] and anchovies [Fig. 22B], also in this case there is not much difference between the mean values of the single units. The price variability of each unit is very high.



22. *Commercial value. (A) Sardine commercial value. (B) Anchovies commercial value*

The graph below [Fig.23] shows the size classes in which the individuals collected each month are distributed. It is possible to notice how the size classes have a typical bell distribution, in which the peak is reached at size 13.5-13.9.



23. *Length distribution*

Thanks to the data collection from the logbook it was possible to record the presence of several species that were subject to by-catches during the sardine fishery. The months in which accessory species have been recorded are April, November, December and August and are *Xiphias gladius* (Linnaeus, 1758), *Seriola dumerili* (Risso, 1810) and *Trachurus trachurus* (Linnaeus, 1758). Only the latter two were fished in August.

In addition to these species, during the sampling in the laboratory, other species were found and are:

- *Scomber scombrus* (Linnaeus, 1758), *Trachurus trachurus* and *Mullus surmuletus* (Linnaeus, 1758) in March;

- Unidentified adult squid, *Pagellus erythrinus* (Linnaeus, 1758), *Trachurus trachurus*, *Trisopterus minutus* (Linnaeus, 1758) and *Serranus hepatus* (Linnaeus, 1758) in May;

- *Sprattus sprattus* (Linnaeus, 1758) in September;

- *Lesueurigobius friesii* (Malm, 1874) in November;

- *Sardinella aurita* (Valenciennes, 1847) in January;

- Unidentified non-adult cephalopods.

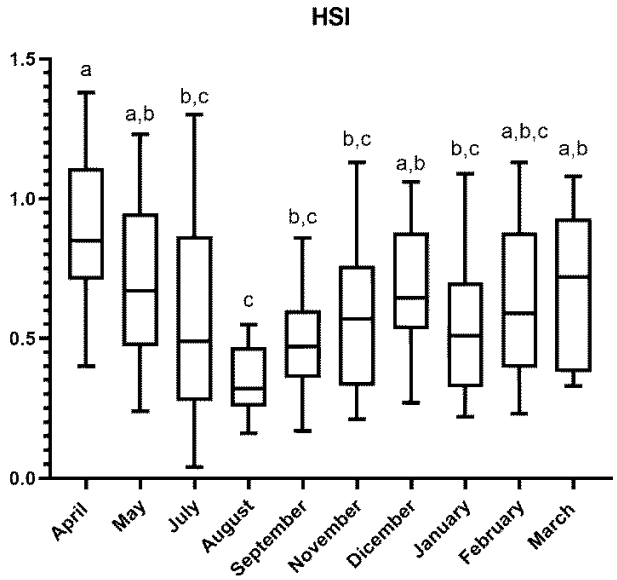
For each species the number of individuals accidentally caught is 2, except for the unidentified cephalopods which were 12.

4.2. Biometrics and general parameters

As regards the body indices, both the hepatosomatic index (HSI) and the Fulton condition factor (K) were calculated.

Hepatosomatic index

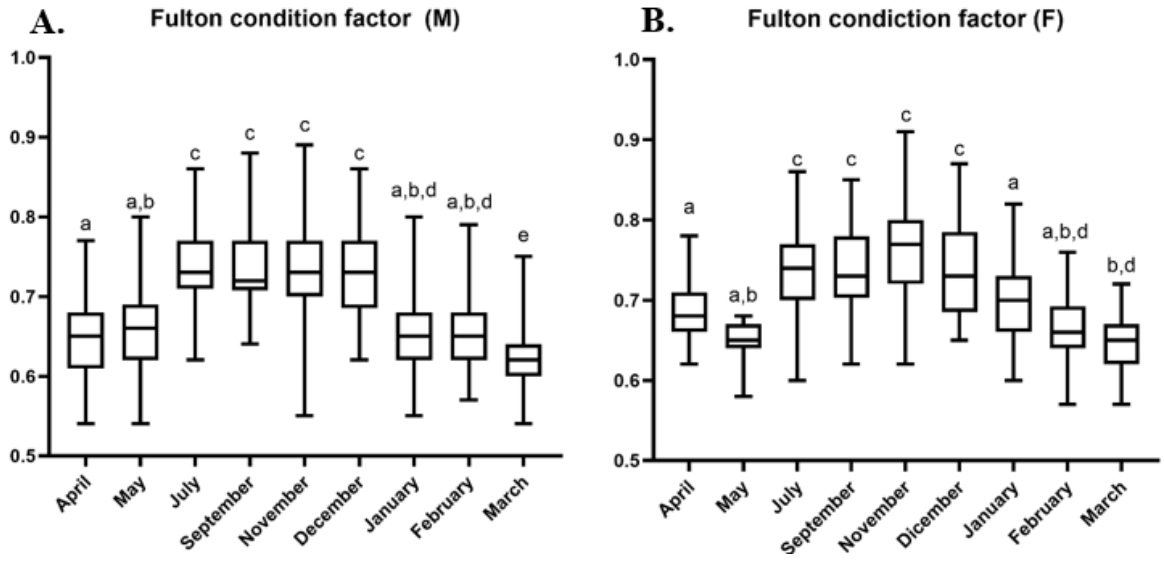
The HSI was calculated for the females sampled and classified each month. From the graph [Fig.24] it is possible to see how the April values are statistically significant compared to the months of July, August, September, November and January. Furthermore, there is a statistically significant difference between August and the months of May, December and March. Beyond the significance ($p > 0.05$), it can be noted that from April there is a decreasing trend that reaches its minimum in August, and then grows back, more or less linearly, till the winter months.



***24**. Hepatosomatic index by month (Min to Max) (p-value < 0.05)*

Fulton condition factor

The Fulton condition factor was calculated for both males [Fig.25A] and females [Fig.25B]. It is possible to see how in females, the values of the Fulton condition are slightly higher than the values of males. Furthermore, in both cases, a statistically significant seasonal trend was evidenced.



25. *Fulton condition factor (Min to Max) for males (A) and females (B) (p-value < 0.05)*

4.3. Pearson's correlation

	Date	Weight	Lenght	% MMs Area	% MMCs Area	N° MMs	N° MMCs	N° Cysts	Healthy tissue	Altered tissue	Altered vessels	Age	Reproductive stage
Date	1												
Weight	**0.20***	1											
Lenght	**0.21***	**0.88*****	1										
% MMs Area	0.14	0.07	**0.18***	1									
% MMCs Area	0.06	**0.21****	**0.27*****	**0.59*****	1								
N° MMs	0.01	0.04	0.14	**0.93*****	**0.62*****	1							
N° MMCs	0.01	0.09	0.13	**0.66*****	**0.52*****	**0.65*****	1						
N° Cysts	0.06	**0.25****	**0.29*****	0.02	-0.09	-0.01	-0.02	1					
Healthy tissue	**0.24****	-0.12	-0.09	**-0.17***	-0.11	**-0.18***	**-0.17***	-0.08	1				
Altered tissue	0.03	-0.15	-0.10	0.02	0.00	0.08	0.11	0.02	**-0.23***	1			
Altered vessels	**0.26*****	0.00	0.07	0.00	0.10	0.09	0.03	0.07	-0.09	**0.40*****	1		
Age	0.06	**0.60*****	**0.55*****	0.16	**0.31*****	**0.18***	**0.17***	0.14	**-0.24***	-0.14	-0.02	1	
Reproductive stage	**0.24****	0.08	0.09	0.15	0.12	**0.17***	**0.21****	0.05	**-0.70*****	0.13	0.06	**0.19***	1

2. Pearson's correlation test. All the bold comparisons are considered significant for a p-value < 0.05

Pearson's correlation highlighted how the area of melanomacrophages, both single (MMs) and centres (MMCs), correlates strongly and positively with length and age (p-value <0.001). Furthermore, the area of individuals is also weakly positively correlated with weight (p-value <0.05).

Noteworthy, the number of cysts also correlates highly and positively with weight (p-value <0.01) and length (p-value <0.001).

Finally, the reproductive stage correlates positively with the number of MMs, with the number of MMCs and with age, and strongly negatively with healthy tissue.

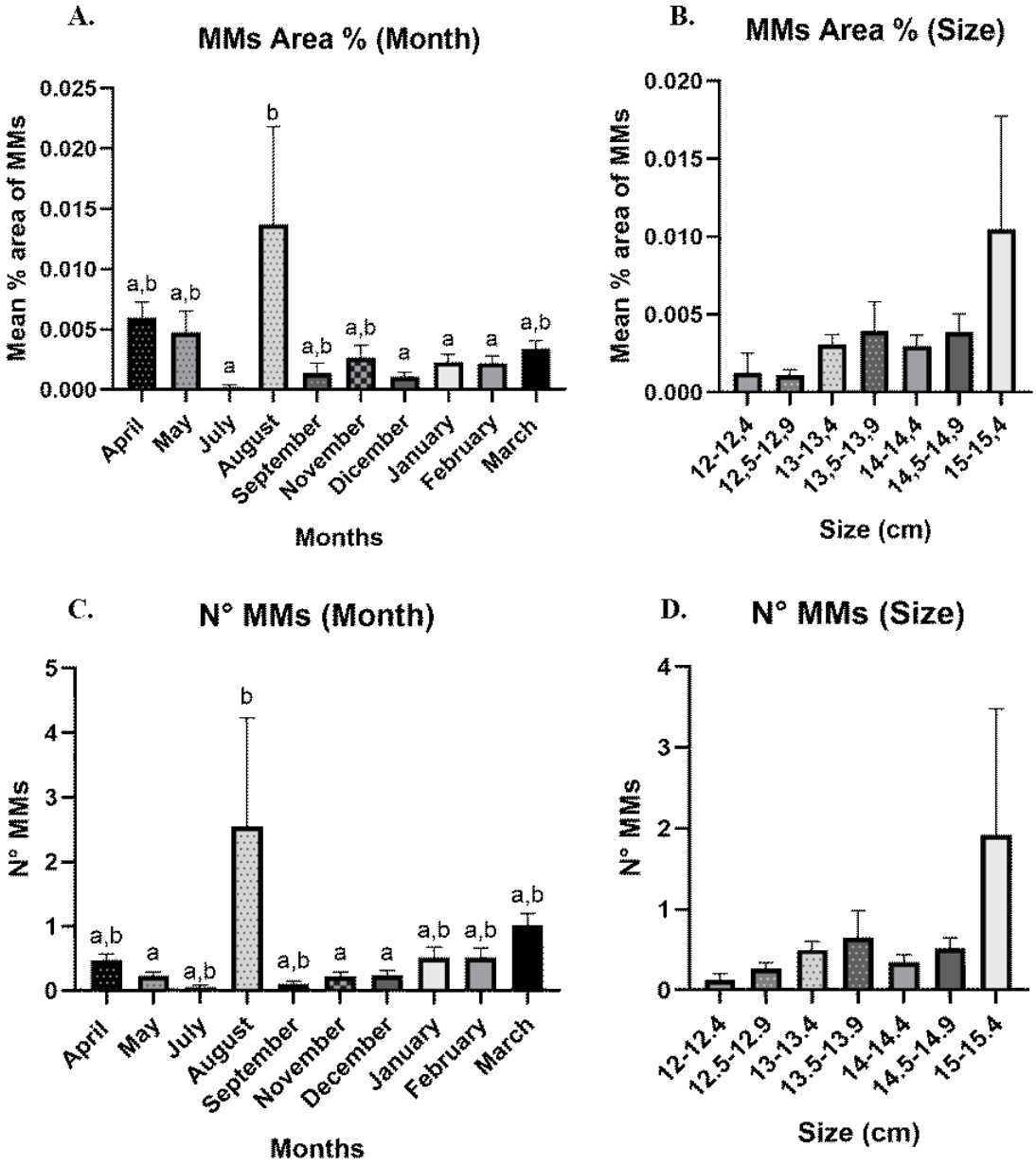
4.4. Melanomacrophages

For both single melanomacrophages (MMs) [Fig.26] and melanomacrophage centres (MMCs) [Fig.25] the number and percentage area were analysed by size and by month on an area of 37492.13 μm.

MMs

In figure 26A it is possible to see how the month of August has the highest value of MMs area and how this is statistically significant with the values of July, December, January and February, which represent the lowest means. Considering the distribution of the MMs area based on size [Fig.26B], no statistically significant differences were evidenced.

As regards the number of MMs per month, only August showed the highest value statistically significant compared to May, November and December, while in the case of the number per size, no statistically significant differences were evidenced.



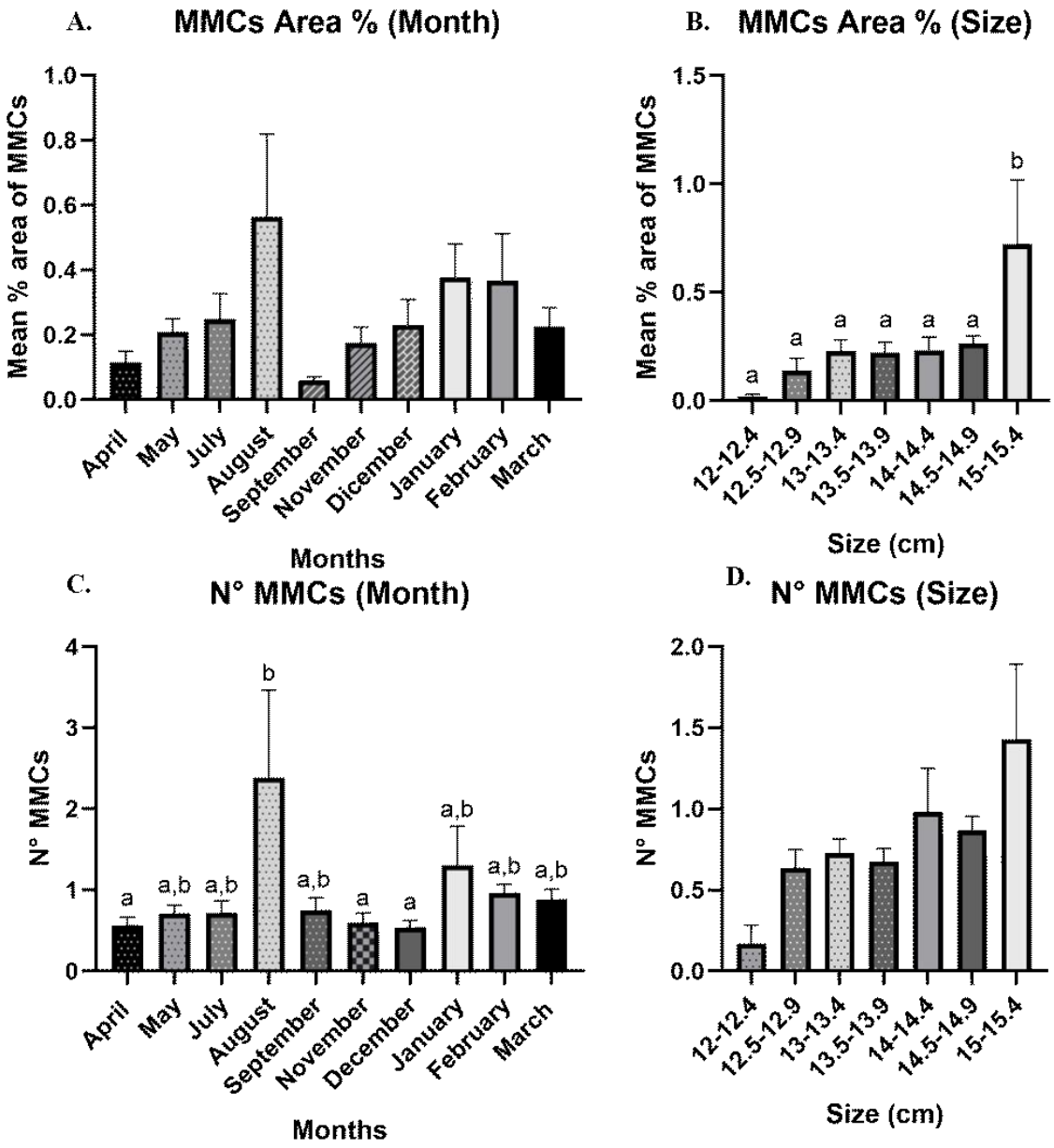
26. *The histograms show mean percentage area (A, B) and number (C, D) of MMs by size and by month (mean ± SEM) (p-value < 0.05)*

MMCs

Considering the MMC area [Fig.27A] per month, no statistically significant differences were detected, even if an increasing trend between April and August and between September and February was evidenced.

Regarding the graph of the area of MMCs per size [Fig.27B], only the last size class, 15-15.4, is statistically higher compared to all the others.

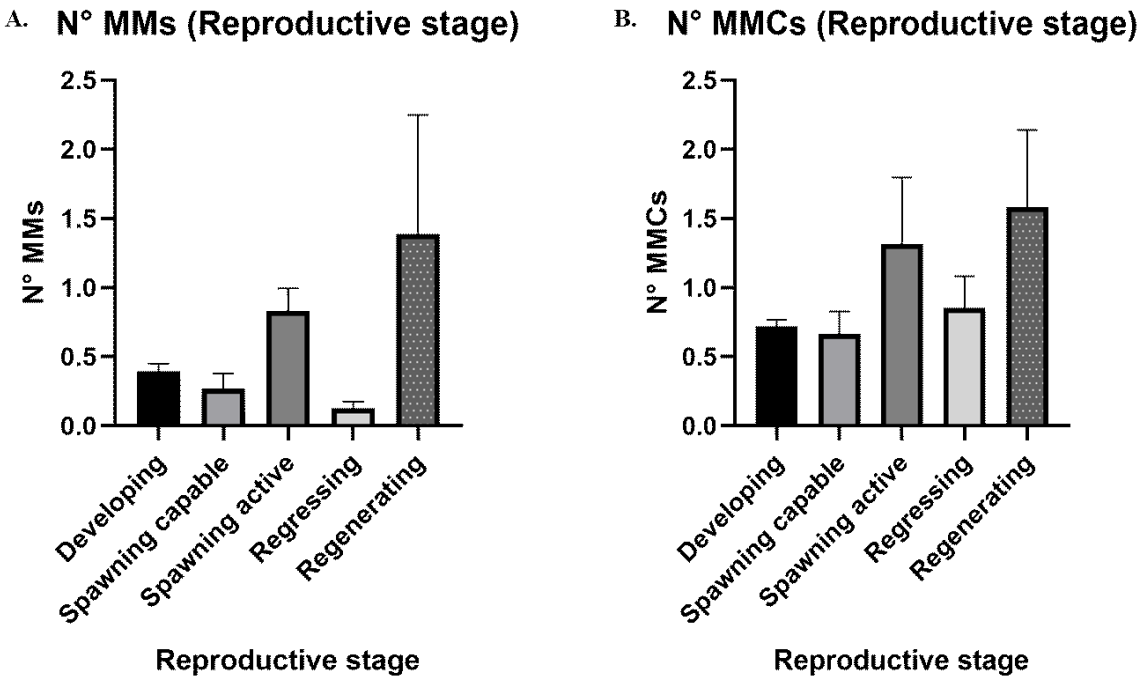
For the number of MMCs per month [Fig.27C], as well as for the number of MMs, the highest value is in August, which is statistically significant compared to April, November and December. Instead, for the number of MMCs based on size [Fig.27D], as well as for the area, there is an increase based on the increase in size, that is not statistically significant.



27. The histograms show mean percentage area (A, B) and number (C, D) of MMCs by size and by month (mean ± SEM) (p-value < 0.05)

Both for the area and for the number of MMs and MMCs based on size. the extreme classes 11.5-11.9 and 15.5-15.9 have been eliminated as they were both represented by a single individual.

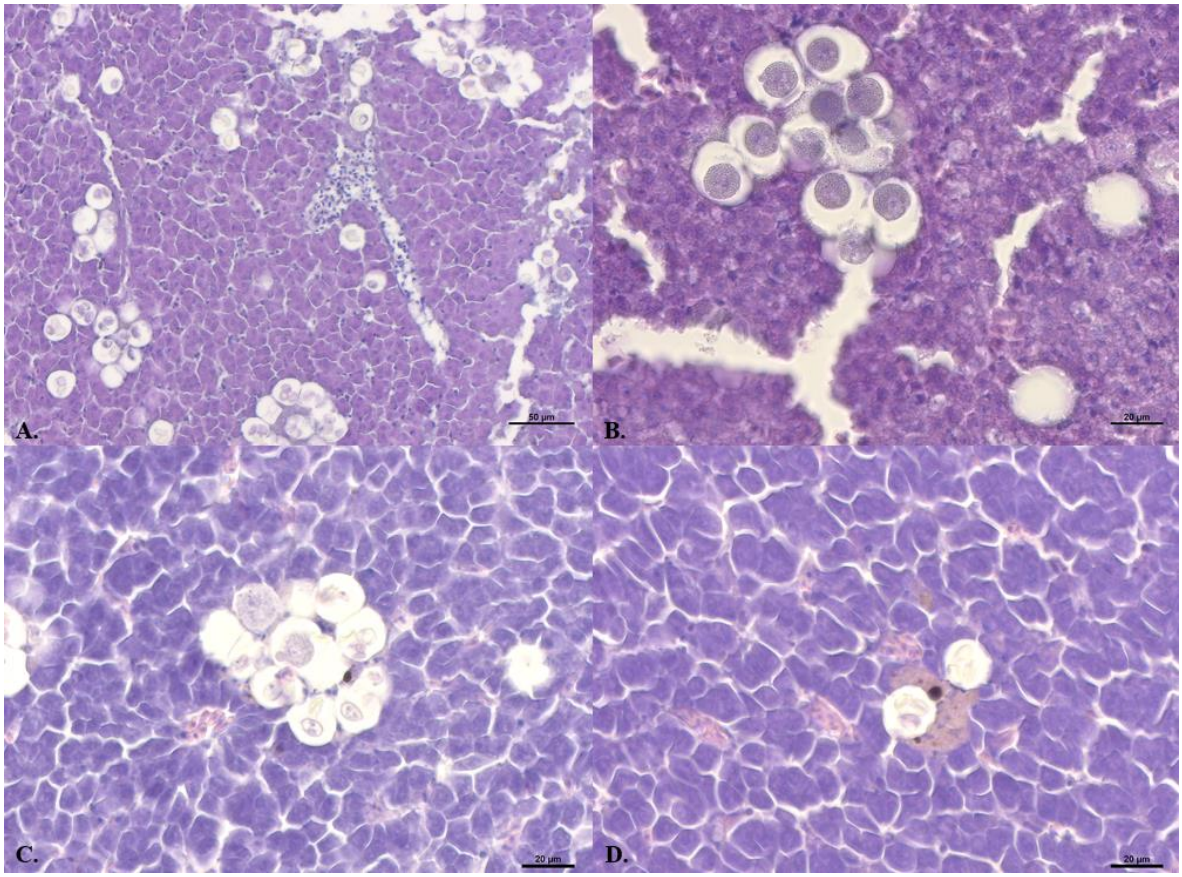
Finally, both the number of MMs and MMCs were related to the reproductive stage [Fig.28]. This highlights how both MMs [Fig.28A] and MMCs [Fig.28B] are more present in the regenerating stage, even if there is no statistically significant difference with the other stages.



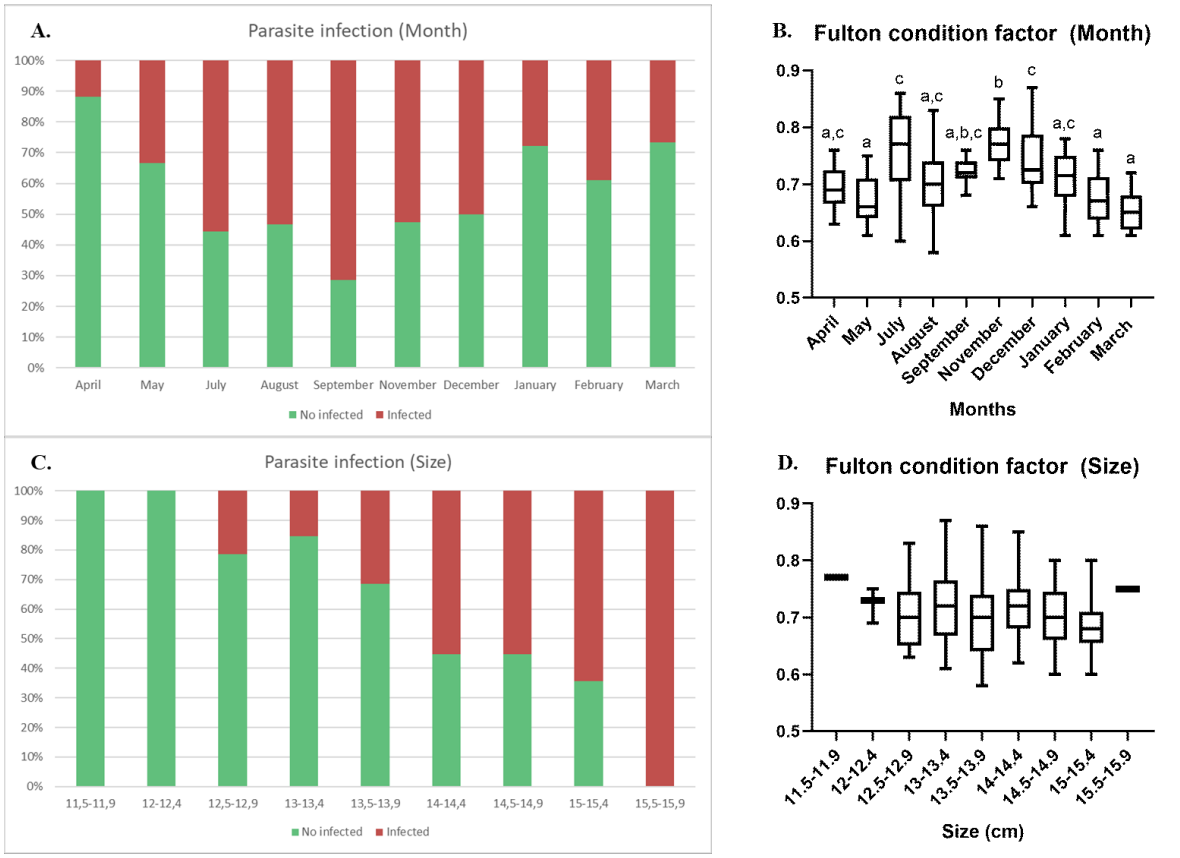
28. *The histograms show the mean number of MMs (A) and of MMCs (B) by Reproductive stage (mean ± SEM) (p-value < 0.05)*

4.5. Parasites

histological slides of females liver were also analysed for the presence or absence of parasites [Fig.29]. The percentage of infected individuals was calculated on the basis of both month [Fig.30A] and size [Fig.30C]. Furthermore, to see if there is a correlation between body conditions and the presence of parasitosis, the Fulton condition factor was also calculated for the same females, both on the basis of the month [Fig.30B] and on the basis of the size [Fig.30D].



29. *Different life stages of parasites. (A) Oocysts with sporozoites [20x]. (B) Oocysts with sporocyst release in the liver parenchyma [40x]. (C) Different stages [40x]. (D) Phagocytosis of an oocyst by an MMCs [40x]*

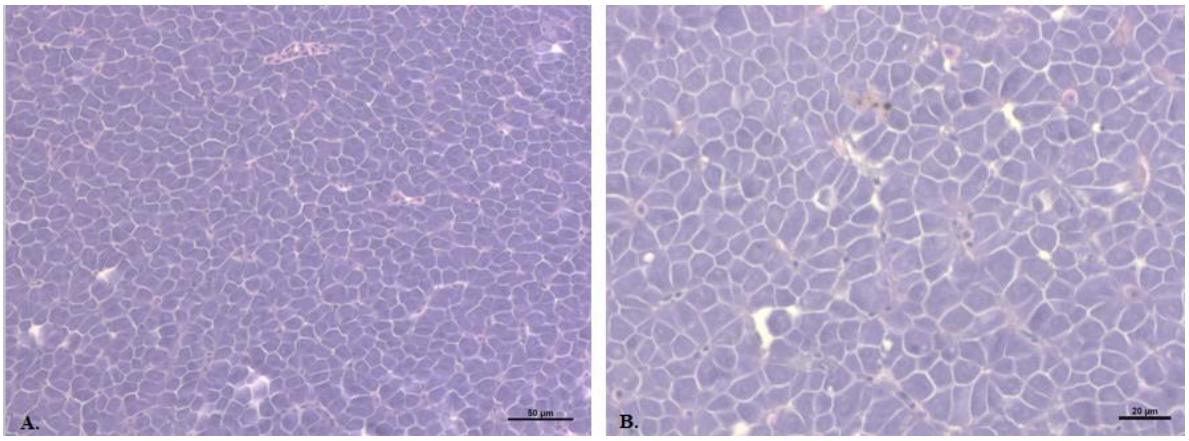


30. *(A) Representation of the infection rate by month. (B) Fulton condition factor by month. (C) Representation of the infection rate by size. (D) Fulton condition factor by size*

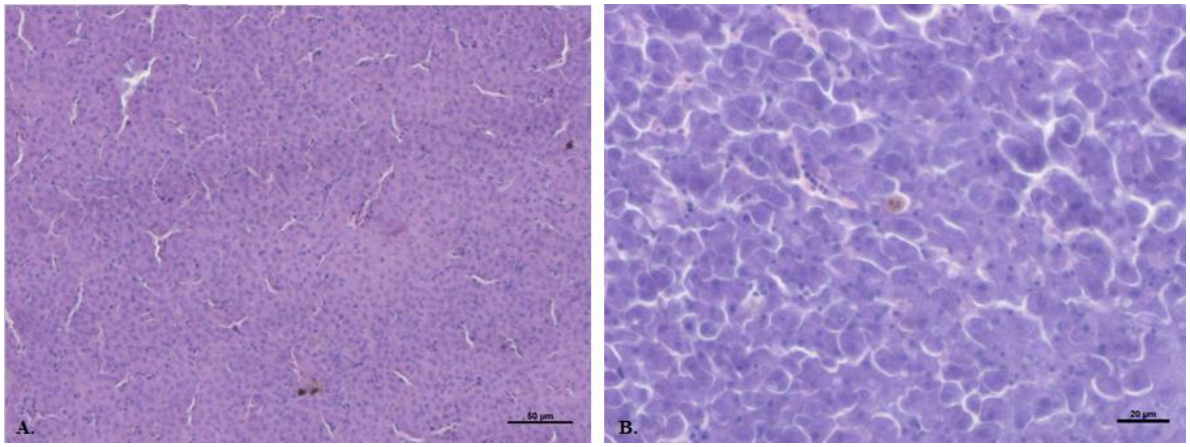
From figure 30A it is possible to see how there is no seasonal trend in the infection of individuals, even if the most affected months are those between July and December. Instead, from figure 30C it can be seen how the number of infected individuals grows as the size increases. As regards the Fulton condition factor, there are no statistically significant differences between the sizes, while the values by month do not follow the trend of infections by month.

4.6. Histological hepatic abnormalities

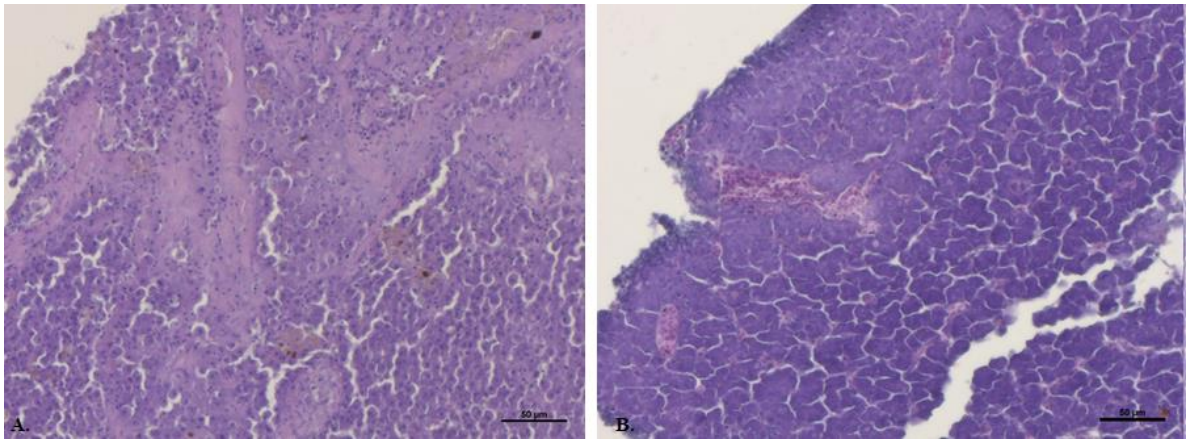
During the histological analyses, in addition to sections of healthy tissue [Fig.31], various abnormalities were found both in the liver parenchyma and in the blood vessels. These abnormalities are: necrosis, with varying degrees of severity based on the alteration of morphology and staining (N1 = loss of cell membranes between hepatocytes [Fig.32]; N2 = alteration of staining [Fig.33]; N3 = total loss of hepatocytes [Fig.34]), vacuolization [Fig.35], thickening of the walls of the blood vessels [Fig.36A], haemolysis [Fig.36B], white blood cells in blood vessels [Fig.37A] and white blood cell infiltration in the liver parenchyma [Fig.37B].



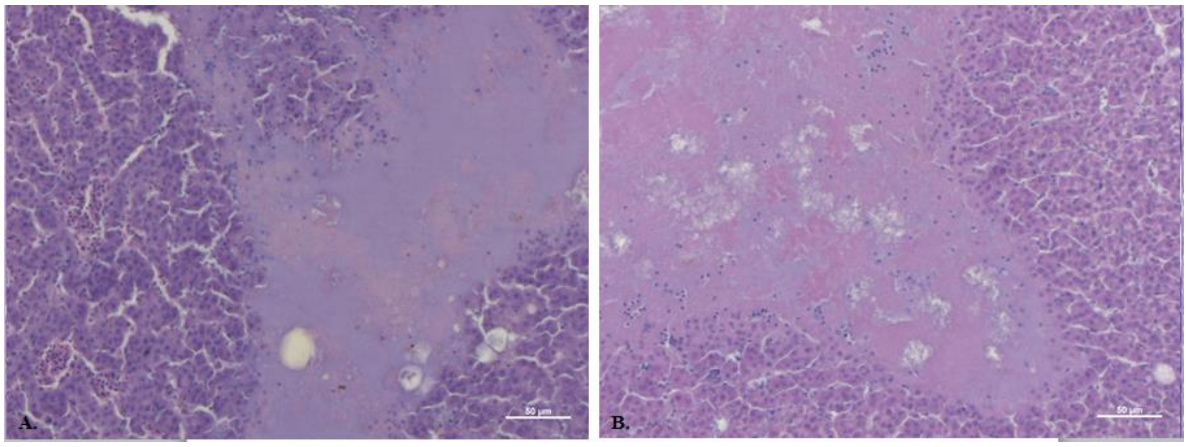
31. *Examples of healthy hepatic tissue (N0) at magnification 20x (A) and 40x (B) magnification*



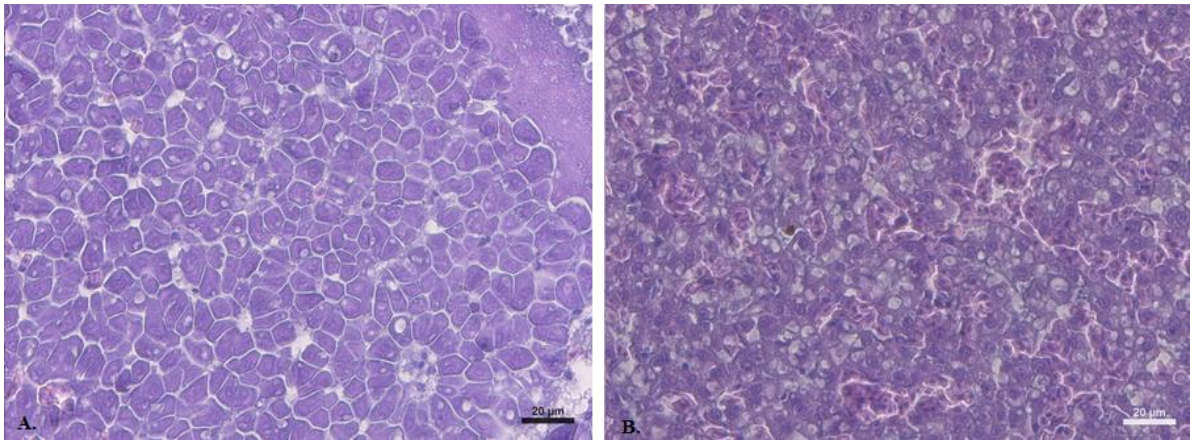
32. *Examples of hepatic tissue with loss of cell membranes and still visible nucleus (N1) at magnification 20x (A) and 40x (B)*



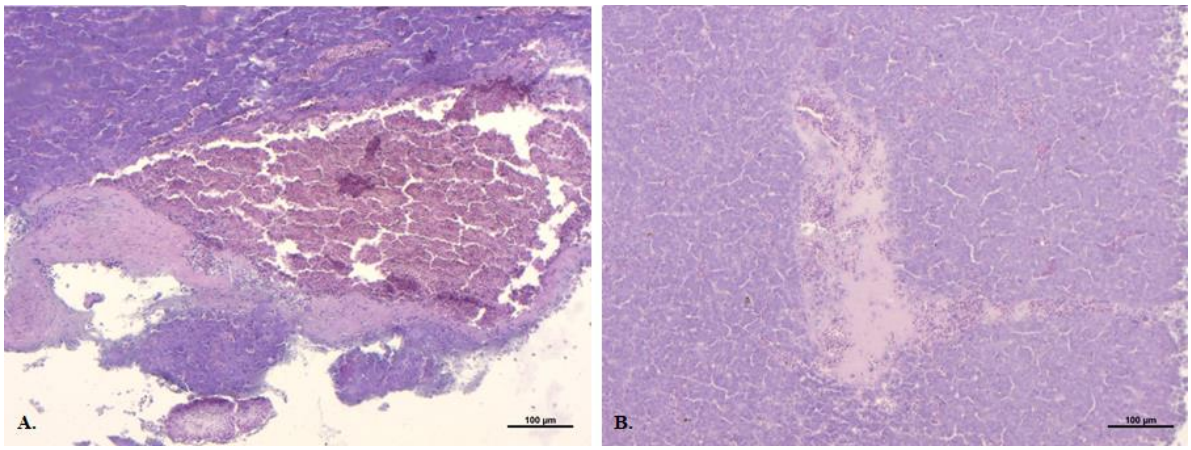
33. *Examples of hepatic tissue with altered staining (N2) at 20x magnification*



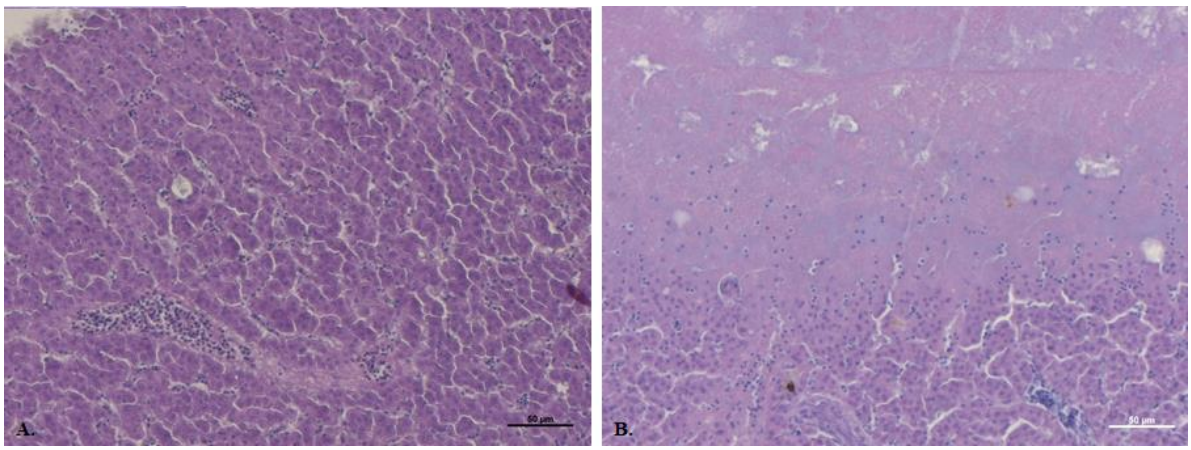
34. *Examples of advanced necrosis of hepatic tissue (N3) at 20x magnification*



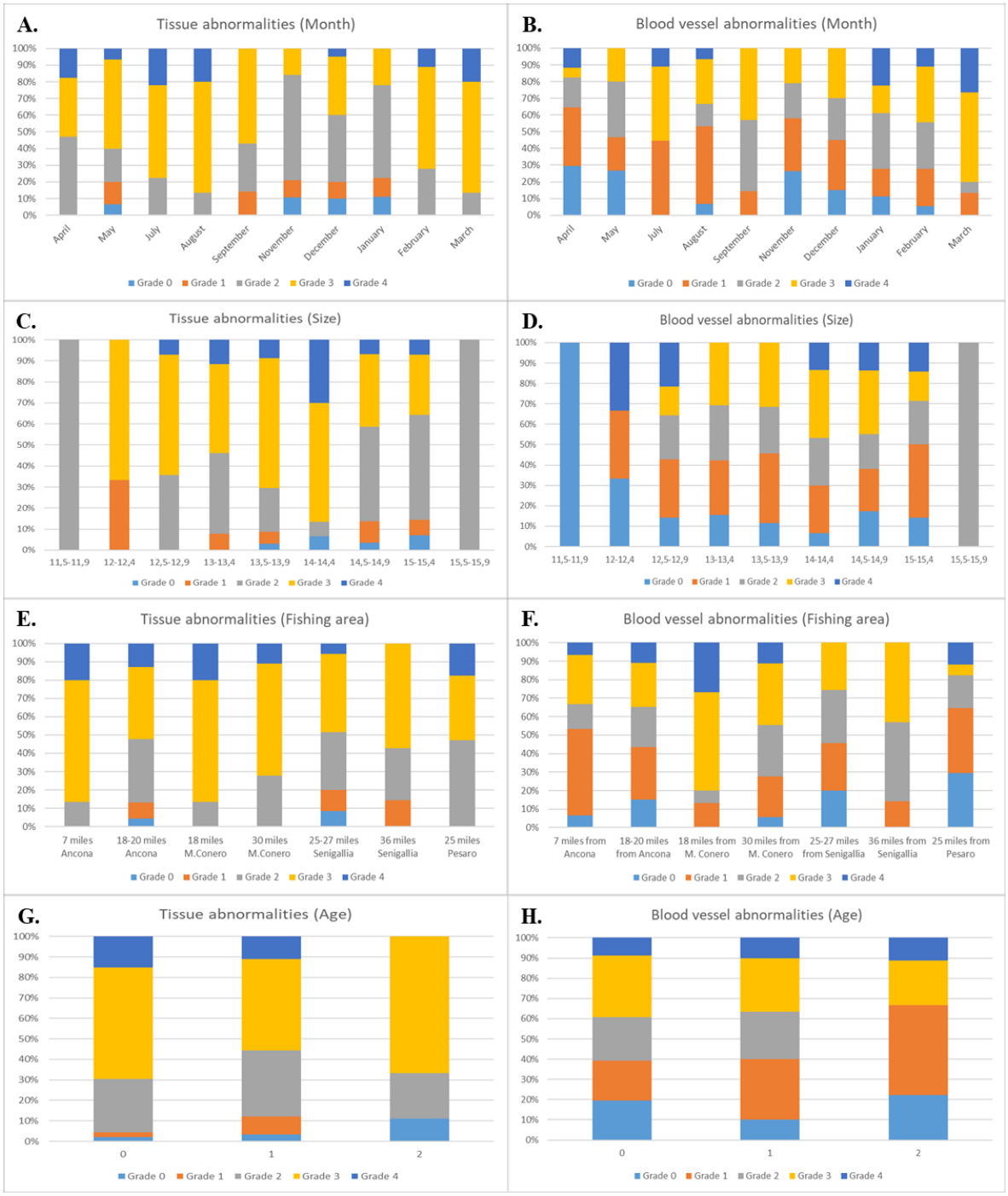
35. *Examples of vacuolation of liver tissue at 40x magnification. (A) Moderate vacuolation. (B) Advanced vacuolation*



36. *Blood vessel abnormalities at 10x magnification. (A) Thickening of the vessel wall. (B) Hemolysis*



37. *Blood vessel abnormalities at 20x magnification. (A) White blood cells in the blood. (B) Infiltration of white blood cells into the tissue*



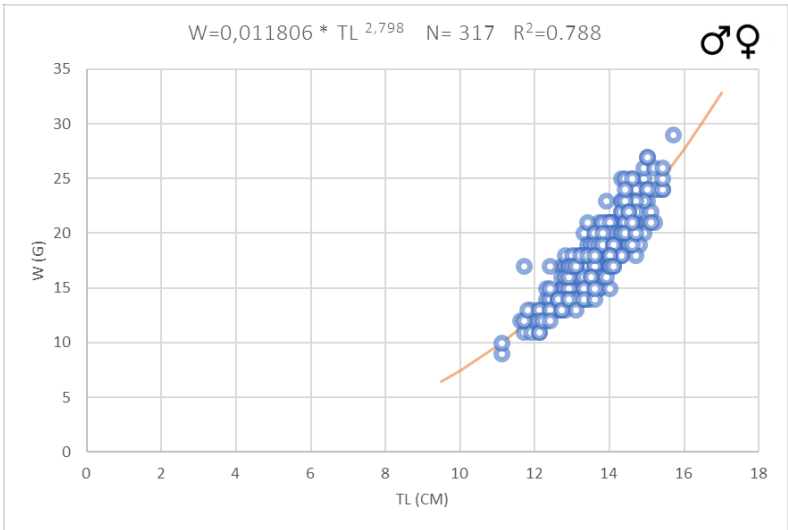
38. *Abnormalities of hepatic tissue and blood vessel. (A) Tissue abnormalities by months. (B) Blood vessel abnormalities by month. (C) Tissue abnormalities by size. (D) Blood vessel abnormalities by size. (E) Tissue abnormalities by fishing are. (F) Blood vessels abnormalities by fishing area. (G) Tissue abnormalities by age. (H) Blood abnormalities by age*

These are the abnormalities according to which the various gradings have been attributed. The gradings were then analysed in association with month [Fig.38A-B], size [Fig.38C-D], fishing area [Fig.38E-F] and age [Fig.38G-H].

From the graphs it is possible to see how the grade 0 related to the abnormalities of the blood vessels is more frequent compared to those of the parenchyma abnormalities. However, in general, the rate of healthy individuals is very low for both grading. It is also possible to see how the grading is influenced neither by the time of year, size or by age. Only for the fishing area it is possible to appreciate how healthy organisms are concentrated in only two areas, 18-20 miles from Ancona and 25-27 miles from Senigallia.

4.7. Otoliths results

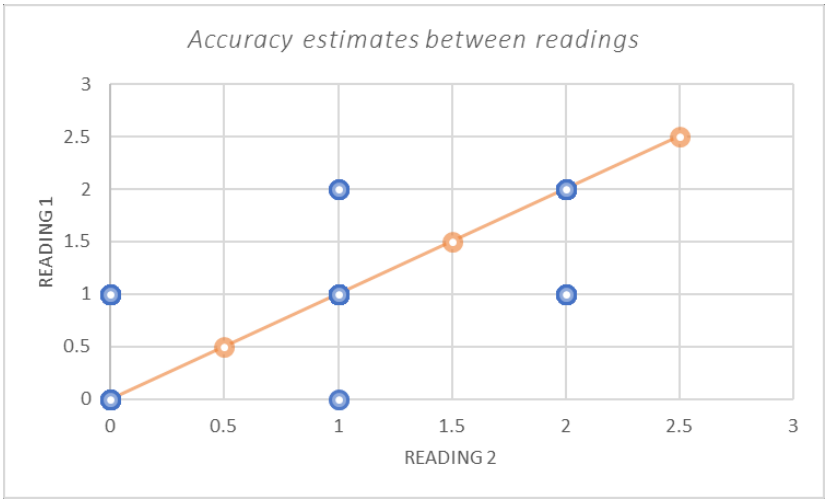
The length-weight relationship was calculated with the logarithm of the formula $W=aL^b$ for the total samples [Fig.37].



39. *Length-weight relationship of total samples ($p < 0.05$)*

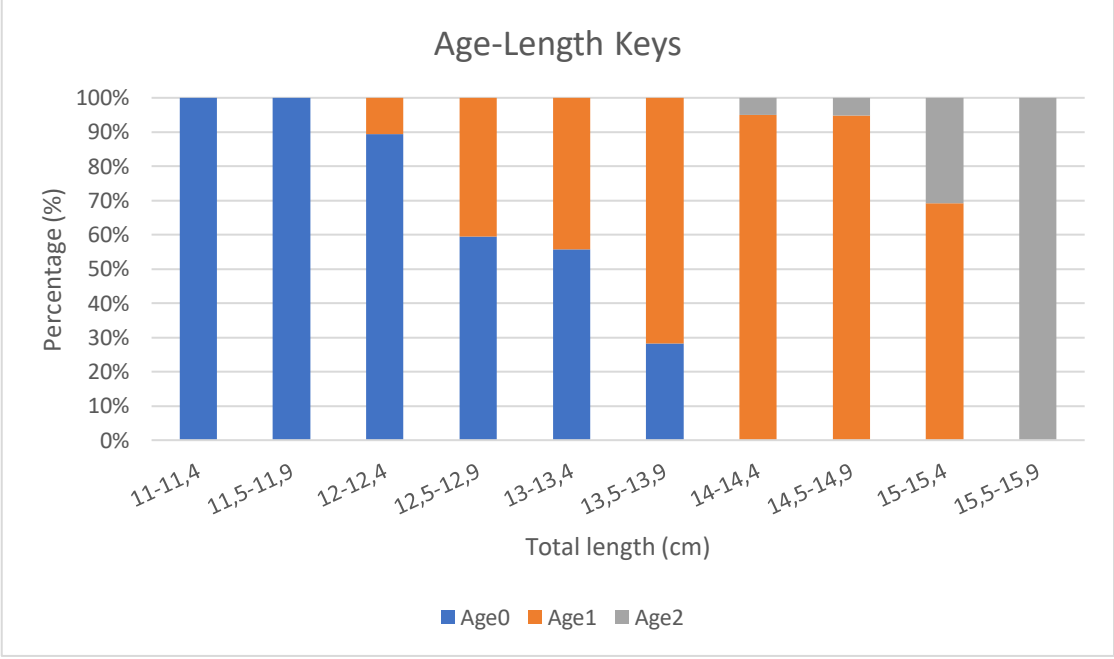
The allometry is negative (i.e. b<3). this mean that either the larger specimens grow more in length or that the smaller specimens are in better nutrition condition.

For the otoliths analyses it was estimated the CV and the APE to determine the accuracy of the age estimates between the two reading [Fig.38].



40. *Accuracy estimate between the two reading (CV = 23%; APE = 16%; Agreement = 75%)*

The representation of the age distribution between the size classes is shown in the figure 39. The graph shows that age increases as the size classes grows.



41. *Age length representation. Each colour corresponds to a given age*

5. DISCUSSIONS AND CONCLUSIONS

The sardine, *Sardina pilchardus* (Walbaum, 1792), is an important commercial resource that also plays a key role in the food chain (Mustac & Sinovčić, 2010). For this reason, fluctuations in the composition of the population can lead to both ecological and economic consequences. Furthermore, the abundance of this species strongly depends on environmental conditions, both directly and indirectly, and this makes it a good bio-indicator for the study of environmental changes (Fernandez-Corredor et al., 2021; Peck et al., 2013).

In recent years the Mediterranean Sea has been subject to rapid degradation, especially since the 20th century (EEA, 2008; Cinnirella et al., 2013; Basilone et. al. 2018), so this study aimed to analyse the health status of the sardine through the analysis of melanomacrophages, an important biomarker (Steinel & Bolnick, 2017), within the liver. This study is important because, despite the commercial value of small pelagic fish, there is little information about their health. In fact, the first work on the suitability of melanomacrophages in clupeids is the one on anchovies by Basilone et al., carried out in 2018.

In this study, what emerges immediately is that the sardine is in very poor health.

The results of this study show that melanomacrophages are positively correlated with biometric factors (length and weight) and age. In particular, with ageing, the number of MMs and MMCs and the area of MMCs increase significantly, while only the area (both MMs and MMCs) increases with length. These results are in agreement with those previously published which showed that with the ageing there is an increase in the density of melanomacrophages (Agius & Roberts, 2003), especially as regards the centres, which represent the response to chronic stress (Agius & Roberts, 2003; Steckert et al., 2018).

Concerning the parasites, the presence of melanomacrophages is not associated with that of oocysts. However, during the analysis of histological sections it was possible to observe how the melanomacrophages perceive the cysts as no-self, since in most cases they were associated with the latter, in attempt to phagocytize them.

In accordance with the data found in the literature (Xavier et al., 2020, 2021; Xavier & Saraiva, 2021) it was possible to determine with certainty the belonging of the parasites to the Coccidia subclass of the phylum Apicomplexa.

Among the three genera of Coccidia that most commonly infect fish are *Calyptospora* (Overstreet, Hawkins & Fournie, 1984), *Goussia* (Labbè, 1896)

and *Eimeria* (Schneider, 1875). In this study it was not possible to determine with certainty the genus of the parasites found in the liver, but the possibility that it is *Calyptospora* was excluded, due to the lack of the typical oocyst projections of this species. Furthermore, the presence of the genera *Goussia* and *Eimeria* in the Clupeids has already been confirmed in several works (Kalfa-Papaioannou & Athanassopoulou-Raptopoulou, 1984; Xavier et al., 2021).

In this study, it was highlighted that the rate of infection depends on the size of the individuals rather than on seasonal variation. Furthermore, since in previous works it has been highlighted how the presence of these parasites determines a worsening of body conditions (MacKenzie, 1981; Xavier & Saraiva, 2021), in the present study the Fulton condition factor has been calculated. What emerged is that animals starting from the size class 14-14.5 cm onwards (the size class 15.5-15.9 cm is represented by only one individual) were characterized by an higher degree of infection and concomitantly by a lower value of Fulton condition factor. Since, also from the Pearson correlation a greater size corresponds to a more intense infection, it can be concluded that, probably, the worsening of the body conditions also depends on the intensity of infection.

Another parameter with which the presence of melanomacrophages has been correlated is the state of integrity of the liver. Melanomacrophages, having both an immunological and physiological function, are normally present within the tissue in low concentrations and tend to increase in the presence of infections and xenobiotic substances (Agius, 1979; Agius & Roberts, 2003). With Pearson's correlation, in fact, it was possible to confirm a significant negative correlation between healthy tissue and the number of MMs and MMCs. Instead, as regards tissue grading and vessel grading, it is possible to see that there is no correlation with the number and area of MMs and MMCs.

Considering both gradings that the number of totally healthy individuals (grade 0) is very low (5 on 153 for tissue grading and 21 on 153 for blood vessel grading), while the severity of the grading varies according to the different parameters considered (month, size and age) and according to the grading. Focusing on healthy individuals it is possible to see how the months in which they are most present are November, December, January and February (considering vessel grading also April). A lower percentage of grade 4 corresponds to these months (except January for vessel grading). during these months, animals heve been caught in the same fishing areas, which are 25-27 miles from Senigallia for May and December and 18-20 miles from Ancona for November and January. The month of July also corresponds to the

fishing area of 20 miles from Ancona. But, this month do not have a healthy individual and, for the tissue grading, it is the month with the highest grade 4. This could be explained by the fact that it is a month in which temperatures are very high and sardines, due to their sensitivity to environmental variations, could be affected by this change (Brossett et al., 2015; Garrido et al., 2016).

Finally, the last considerable parameter with which melanomacrophages have a significant correlation are the reproductive stages. In particular, the latter correlate significantly and positively with the number of MMs and MMCs. Focusing on this result it emerged that the number of melanomacrophages (both MMs and MMCs) increases in the spawning active and regenerating stages. As for the greater number of melanomacrophages during the active spawning phase, this could be explained by the fact that during the reproductive period the energies are assigned to reproduction and not to the organism, thus leading to an increase in stress levels. In support of this, in the regressing phase, outside the reproductive activity, the number of melanomacrophages is lower.

Previous studies demonstrated that during vitellogenesis, the liver detoxifies itself by transporting pollutants to the gonad through the transport of vitellogenin (Pitt et al., 2018; Tye & Masino, 2019). This process could

explain the high number of melanomacrophages found in the regenerating phase during which the hepatic synthesis of vitellogenin and its transport to the gonad is not yet started.

The possibility of the presence of pollutants in the liver of the sardine is supported by works found in the literature in which the same abnormalities in the liver tissue have been associated to the presence of heavy metals (Mohamed, 2008; Younis et al. 2013). In this case, the lack of correlation between grading and the presence of melanomacrophages can be explained by the fact that the conditions of the liver are now too altered. Another hypothesis for which there is no correlation, is that swernereveral factors such as the temperature, which strongly influences on the sardine conditions (Fernandez-Corredor et al., 2021), are not involved in the activation of melanomacrophages.

For this reason, it would be advisable to conduct an analysis that aims to study the presence of heavy metals and the expression of *Heat Shock Proteins* (HSP) as a marker to verify the stress caused by temperature rising (Werner et al, 2007; Oksala et al., 2014).

Furthermore, it is appropriate that the analysis of heavy metals is carried out not only on the liver but also on other organs, such as the gonads, as already in other studies the negative effect of heavy metals on the reproductive cycle

has been reported (Authman et al., 2015; Taslima et al., 2022), and muscle, which represents tissue subject to human consumption.

Finally, as far as parasites are concerned, it would be appropriate to deepen the studies regarding their life cycle and their effects on marine organisms, given the serious lack of information in this regard, to determine the consequences that their infection may have on the stock.

This is the first study that evaluates the health of the sardine, especially in the Adriatic Sea. The results found have highlighted how dramatic the situation is and how the health of this species is influenced by various factors (infections, pollutants and climate change).

This study could therefore represent the basis for the establishment of annual and more targeted monitoring.

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