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DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale
Biologia Marina

Riproduzione della sardina (*Sardina pilchardus)* in Adriatico, quanto ne sappiamo? Uno studio integrato che valuta la crescita e la riproduzione in relazione alla taglia di cattura

The reproduction of sardine (*Sardina pilchardus)* in the Adriatic Sea, what do we know? An integrated study evaluating age growth and sexual maturity related to catch sizes

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A mia madre e a Val.

RIASSUNTO

La sardina europea, *Sardina pilchardus*, è un piccolo pesce pelagico che gioca un ruolo chiave nei processi ecologici dei sistemi marini. È, inoltre, una risorsa biologica molto importante per il commercio adriatico, arrivando a costituire il 15% dello sbarcato totale. Ciò nonostante, la maggior parte degli studi su questa specie sono stati condotti sulla costa orientale dell'Adriatico. La mancanza di informazioni per la costa occidentale crea, dunque, delle lacune significative non solo sulle conoscenze della biologia della sardina, ma anche per la corretta gestione della pesca.

Pertanto, questo studio pone per la prima volta l'attenzione sulla caratterizzazione del ciclo riproduttivo della sardina in relazione all'età e alla pesca nell'Adriatico occidentale. Grazie all'analisi microscopiche sulle gonadi femminili, sono stati quindi descritti gli stadi di riproduzione nei diversi mesi. Inoltre, grazie alla lettura degli otoliti presso il CNR di Ancona, è stato possibile determinare l'età di ogni individuo. Infine, grazie alla collaborazione con l'Università di Catania, è stato valutato lo sforzo di pesca e il valore commerciale delle sardine, oltre che la distribuzione spaziale degli individui nelle diverse zone di pesca. Oltre a definire il ciclo riproduttivo, dalle analisi svolte è emersa la presenza di anomalie nelle gonadi femminili,

le cui cause potrebbero risiedere nell'aumento delle temperature o presenza di inquinanti. Dai risultati ottenuti, potrebbe essere fondamentale svolgere un monitoraggio annuale così da osservare possibili fluttuazioni nella popolazione e comprendere cause e conseguenze di tali anomalie.

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1. INTRODUCTION

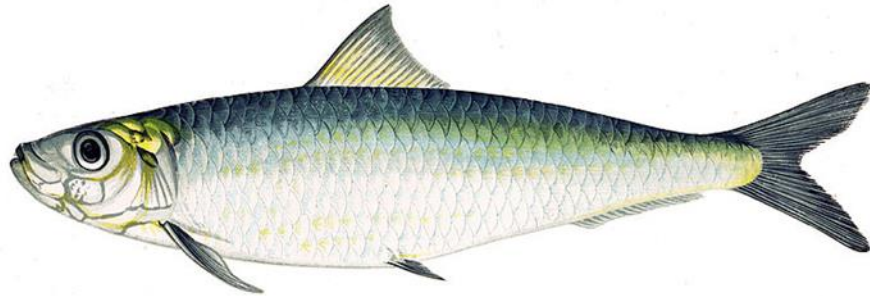## *1.1. Biology and ecology of Sardina pilchardus (Walbaum, 1792)*

Figure 1. *Sardina pilchardus* **(Walbaum, 1792).**

Taxonomic classification:

Phylum: *Chordata*

Subphylum: *Vertebrata*

Infraphylum: *Gnathostomata*

Class: *Actinopteri*

Subclass: *Teleostei*

Oder: *Clupeiformes*

Family: *Clupeidae*

Genus: *Sardina*

Species: *Sardina pilchardus*

The sardine or European sardine (*Sardina pilchardus*, Walbaum, 1792) is a member of the family Clupeidae (Figure 1). It has a fusiform, laterally compressed body and homocercal caudal fin. The mouth is turned upwards, with the upper jaw shorter than the lower one. This is a characteristic feature that distinguish sardines from the similar anchovy (*Engraulis encrasicolus* (Linnaeus, 1758)), which instead has a longer upper jaw. The whole body, except the head, is covered with deciduous scales. The operculum has 3 to 5 distinct striae and the eye is large. The dorsal fin is central and very short. The ventral fins are rather small and located in the ventral part, in correspondence with the centre of the dorsal fin and it is devoid of spiny rays likewise all the other fins. The dorsal is gray-blue, while the ventral part is white and there may be small dark spots on the sides. Sardines' distribution extends from the North Sea to the Senegal coast in the Atlantic waters, as well as in the Mediterranean Sea (Parrish et al., 1989), included the Adriatic Sea (Figure 2).
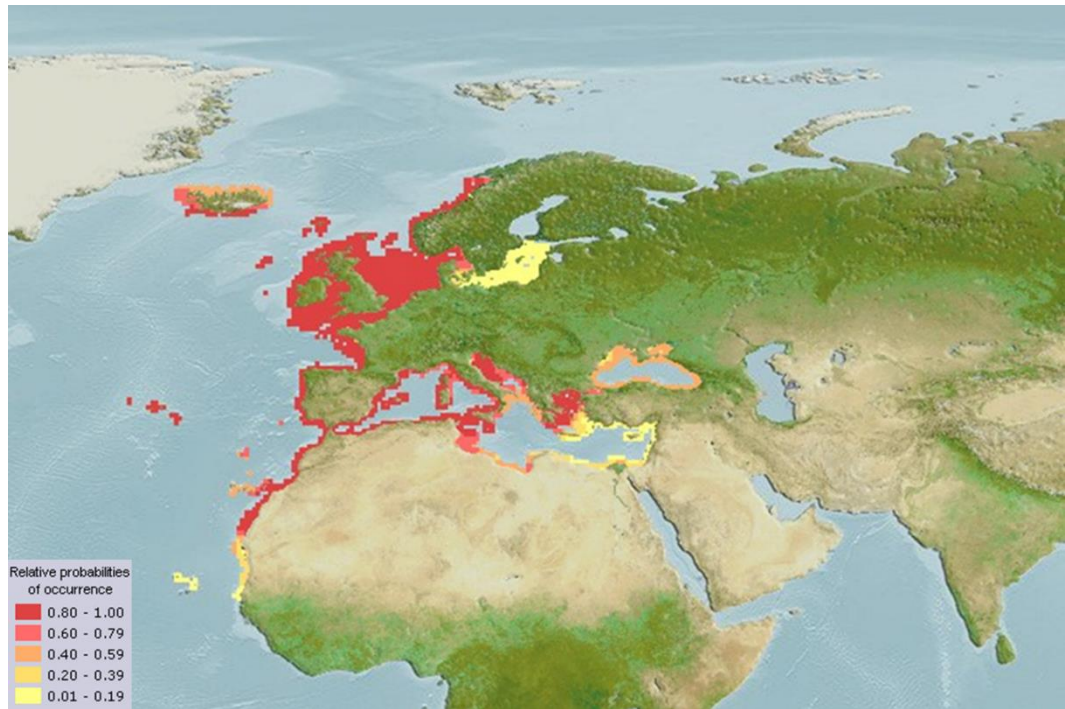
Figure 2. Spatial distribution of *Sardina pilchardus* (Walbaum, 1792) (www.fishbase.org).

European sardine is a gregarious fish living in temperate waters of the continental shelf (Iglesias et al., 2003; D'Elia et al., 2014). *Sardina pilchardus* represents one of the most important small pelagic fish resources in the Mediterranean Sea, in terms of biomass and commercial interest and, according to FAO, its landing represented the 15% of total Mediterranean production between 2016 and 2018 (FAO, 2020). The sardine plays a key role in ecological processes, occupying an important intermediate trophic level in pelagic ecosystems (Bakun, 2006; Rumolo et al., 2016). It is involved in the energy transfer from the bottom to the top of the trophic web. The sardine, indeed, is an important prey for different pelagic species such as seabirds

(Navarro et al., 2009; Cury et al., 2011), tunas (Navarro et al., 2017), cetaceans (Gómez-Campos et al., 2011), demersal predators (Recasens et al., 1998; Mellon-Duval et al., 2017; Saraux et al., 2019), and in turn it preys on several planktonic species, both zooplankton and phytoplankton. Although information on the feeding behavior of sardines is very scarce in the Adriatic Sea, two different feeding behaviors were observed in the Clupeidae family: particulate feeding (selective) and filter feeding (non-selective) (James, 1986). Sardines are capable of switching from one type to another based on the feeding conditions. In fact, the filter feeding is used when small particles are present, while the particulate feeding is used when the particles available are large (Garrido, et al. 2007). Sardines are omnivorous, their prey comprise copepods, decapod larvae, mysid and copepod eggs in particular (Hure and Mustać, 2020). According to a 1963 study by Vučetić carried out during the period April-October in the central Adriatic, the feeding activity of sardines takes place mainly during sunset, and then decreases during the night. In winter, however, feeding was seen to occur early in the day, due to the lack of light in the season. The importance of light is confirmed by night fishing: the best catches occur on full moon nights (Morello & Arneri, 2009) or using a light source. It has also been seen that sardine, like all pelagic fish, carries out vertical migrations (even at the larval stage) for various reasons, including

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escaping predators and feeding (Santos et al., 2004 & 2006; Ribeiro et al., 2005). In fact, they move upwards, in conjunction with the migration of plankton, to feed and downwards to escape predators (Zwolinski et al., 2007).

1.2. Reproduction

1.2.1 Reproduction in teleost species

Vertebrate reproduction is a complex mechanism regulated by the hypothalamus-pituitary gland- gonadal axis (Figure 3). The hypothalamus produces the hormone GnRH (gonadotropin-releasing hormone) which binds to the membrane receptors of pituitary cells. This bond stimulates the transcription and translation of the luteinizing hormone (LH) and follicle stimulating hormone (FSH), two pituitary gonadotropins that are released into the bloodstream to reach the gonads. Under the stimulation of these two hormones, gametogenesis and the production of estrogen, testosterone and progesterone begins (Carnevali et al., 2019).
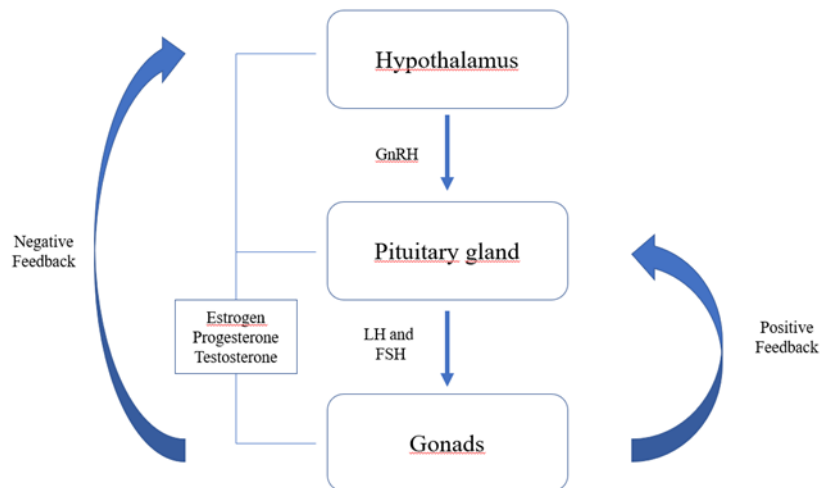

Figure 3. Diagram of the hypothalamus- pituitary gland- gonadal axis.

In females, the FSH receptors is located in the granulosa cells membrane, while LH on theca cells. In the male, however, FSH has receptors on Leydig cells and LH on Sertoli cells. All teleosts have a similar gonadal development and show a reproductive cycle with different stages, that are described in a conceptual model by Brown-Peterson in 2011 (Figure 4) and briefly described as follow:

- Immature, characterized by oogonia and primary growth oocytes only, with no atresia;

- Developing, where more cell types occur, such as primary growth oocytes, cortical alveolar, primary and secondary vitellogenic oocytes and few cases of atresia is possible;

- Spawning capable, which present tertiary vitellogenic oocytes, some hydrated oocytes, atresia and postovulatory follicle (POF);

- Actively spawning, considered as the final part of the spawning capable phase when the oocytes undergo late germinal vesicle migration and breakdown and hydration of occurs;

- Regressing, characterized by high frequency of atresia events and POF, cortical alveolar and vitellogenic oocytes could be still detectable;

- Regenerating, with only oogonium and primary oocytes and advanced atretic follicles and degenerating POF.
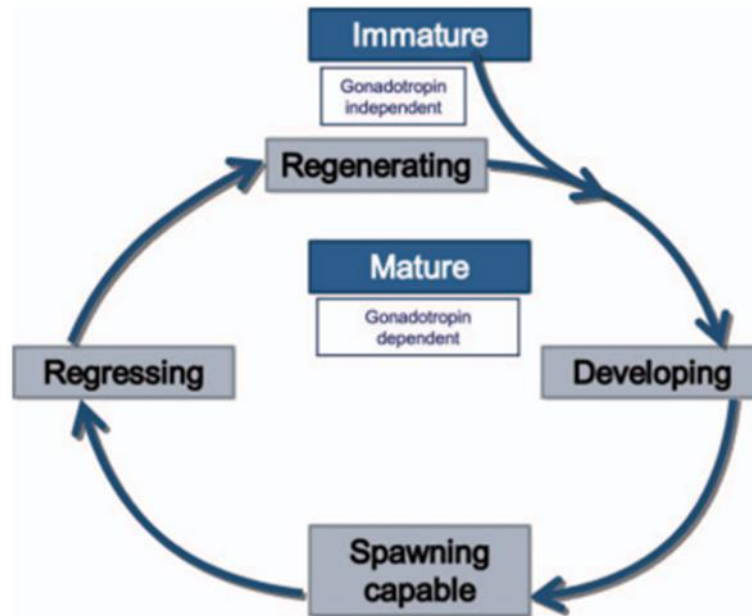

Figure 4. Conceptual model of fish reproductive phases (Brown-Peterson et al., 2011).

1.2.2 Oogenesis

Oogenesis is the process through which, in mature females, oogonia mature into oocytes, reaching a 100-200 times larger size than the initial cell (Wootton & Smith, 2014). The simultaneous maturation of all oogonia during the reproduction season leads to all the oocytes at the same developmental stage at a given time and it is identified as synchronous maturation. This strategy is typical of all these species that reproduce only once a year or once in their lifetime (such as salmonids) (Mylonas et al., 2000). Differently, when the oogonia develop at different time, we refer to it as asynchronous maturation. Asynchronous fish, (*Danio rerio,* (Carnevali et al., 2019)), are capable of ovulating on a regular basis, sometimes every day, over a prolonged period during which in the ovary is characterized by all oocyte's stages. A synchronous maturation in groups is also possible, as for *Sardina pilchardus* (Walbaum, 1792), in this species batches of oogonia mature at different times, in this way it is possible to have more ovulations within the reproductive season.

During oogenesis there are two phases of growth: the first one is a slow growth in which the synthesis of macromolecules (such as mRNA) and proteins takes place by the oogonium itself, this phase is regulated by FSH

(Vacher, C., et al., 2000); the second phase, regulated by LH, is characterized by a fast growth due to the uptake of macromolecules produced by the liver (Figure 5).
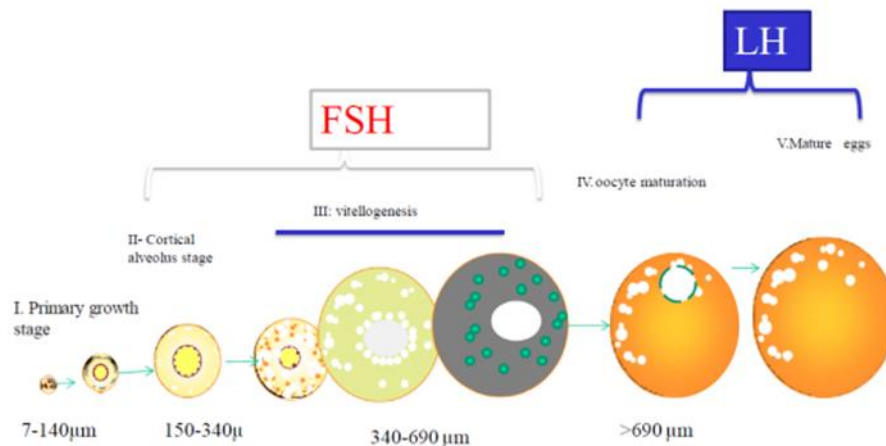
Figure 5. Oogenesis stages.

During the oocyte's maturation, two types of somatic cells are involved as essential elements for a proper completion of the maturation process. These two somatic cells are the granulosa and theca cells (Nagahama et al., 2008). These two types of cells with oocytes and basal lamina form a complex, called ovarian follicle. The granulosa cells are the cells in direct contact with the oocyte, they form a single cell layer surrounded the oocyte. The external layer is composed by the theca cells which are separated from the granulosa cells through a basal lamina and an extracellular matrix (Wootton & Smith, 2014; Lubzens et al., 2010). The first stage of follicle maturation leads to the

formation of the primary oocyte. The oogonium enters the meiosis and begins the prophase to stops after reaching the diplotene stage (Le Menn et al., 2007). In this first phase the chromosomes are not condensed and are called "brush-like". Furthermore, the nucleoli inside the nucleus are still very visible, reason why this phase is named perinucleolar (Figure 6A).
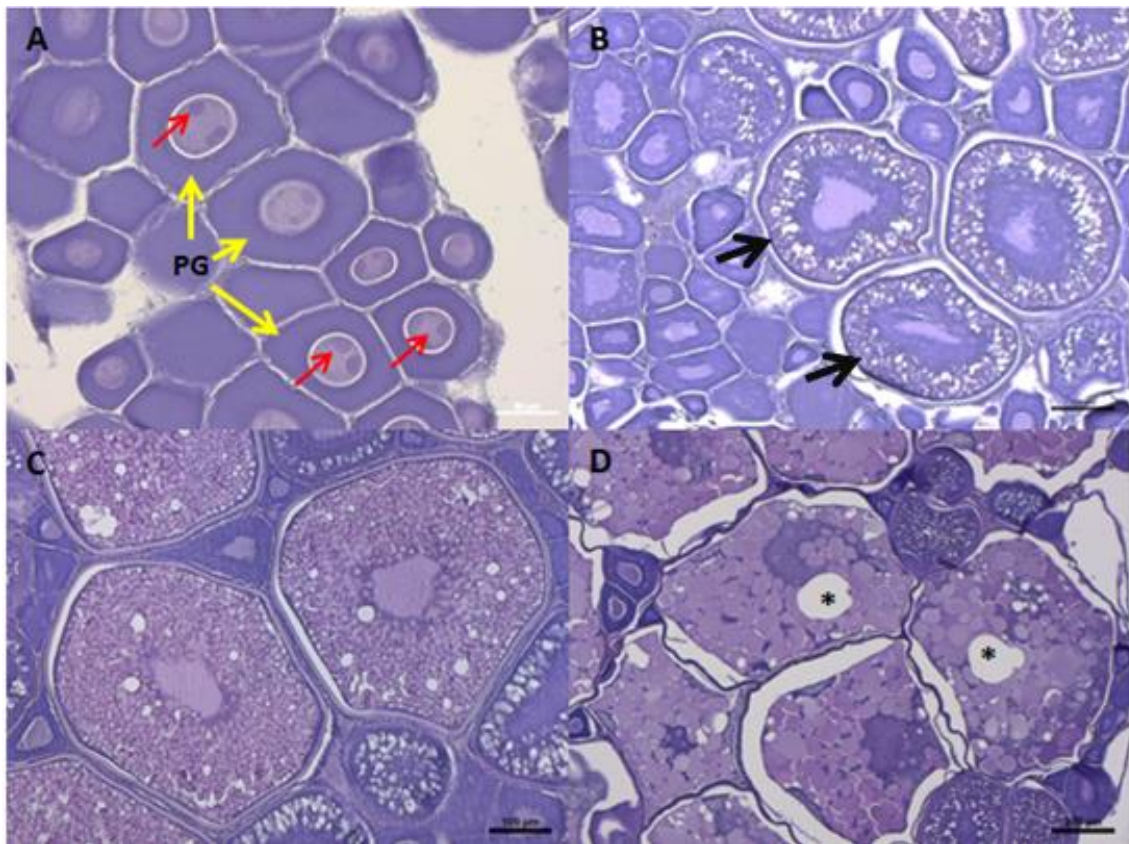
Figure 6. Histological images of *Sardina pilchardus* ovarian illustrating the different stages of females' reproductive cycle. (A) Perinucleolar stage; (PG) (yellow arrow), primary growth oocytes with the nucleoli (red arrows); (B) lipid droplets in the oocytes (black arrow); (C) Vitellogenic oocytes; (D) Oocytes with vitellogenin in coalescence with large lipid drop (asterisk).

During this phase, the first division does not occur, however, the oocyte grows and becomes bigger due to mRNA and proteins production. Some of

the transcript mRNAs are packaged and used in the early stages of oogenesis and embryonic development, while others are readily translated. In this phase, within the oocyte there are also the Balbiani Bodies, which are non-membrane bound compartment of the cytoplasm in which ribosomes and mRNA accumulate (Charitonidou et al., 2022). From the onset of the meiosis, the oocyte and granulosa cells come into close contact thanks to the formation of microvilli which allow the exchange of hormones and macromolecules. Finally, after the perinucleolar phase, within the ooplasm, also occurs the formation of the cortical alveoli, vesicles produced by the Golgi apparatus and retained in the oocyte (Carnevali et al., 2019). The contents of these vesicles, rich in proteins and carbohydrates, will then be released out the ooplasm, at the moment of fertilization, to prevent polyspermy (the entry of other spermatozoa in the fertilized oocyte). The second step of the maturation process is characterized by the appearance of lipid droplets in the oocyte (Figure 6B), these are composed of neutral fats (initially they are important sources of energy for females) produced by the liver. As they migrate towards the nucleus, these lipid droplets fuse to form one large central lipid drop. This lipid drop is important because it allows the egg to float in the water column and represents an energy resource for the development of the embryo. Also, during the slow growth phase, the zone radiata or pellucida is formed, this is

an external covering of protein-nature, located between the plasma membrane of the oocyte and the granulosa cells (Corriero et al., 2004). The proteins that form the zone radiata derive both from the oocyte itself and from the liver (Babin et al., 2007). In the latter case, the proteins reach the oocyte through the bloodstream where they are internalized thanks to the formation of endosomes. The above-mentioned processes take place under the control of the FSH (Babin et al., 2007). At this point vitellogenesis begins, a rapid growth phase under the control of the LH (Babin et al., 2007). During the vitellogenesis, the radiated zone thickens to protect the oocyte from external interference (Lubzens et al., 2010). Vitellogenin is a lipo-glyco-phosphoprotein, synthesized by the female liver that reaches the oocyte through the bloodstream (Babin et al., 2007). Vitellogenin is the precursor molecule of the yolk and enters the oocytes through the capillaries of the theca cells. Successively it crosses the granulosa cells layer and finally reaches the receptors of oocyte plasmatic membrane. Once the binding has occurred, the vitellogenin is endocytosed (Figure 6C) and the receptor detaches itself to return to the plasmatic membrane where it can bind to new vitellogenin molecules. The vitellogenin detachment from the receptor occurs thanks to cathepsins, proteolytic enzymes contained in lysosomes (Kagawa et al., 2013). Indeed, vitellogenin is broken down into its minor components:

lipids, phosphates and proteins. The final components present in the ooplasm are: phosphovitin, lipovitellin, β-component and C-terminal peptide. The protein part of the vitellogenin is dismantled into single amino acids to facilitate their storage as a reserve and also to increase the osmotic concentration within the oocyte. Thus, after the accumulation of a proper amount of "vitellogenin-components", the water can enter leading to the hydration phase (Figure 6D) (Kagawa et al., 2013). At the end of the vitellogenesis, maturation begins and determines the resumption of meiosis in addition to the recall of water in the oocytes (which occurs not only by osmosis, but also thanks to the aqueporins). Meiosis continues up to metaphase II to stop again and be concluded only after the fertilization (Kagawa et al., 2013). An important role is played by the progesterone hormone (produced by somatic cells), whose receptor is located in the outer membrane of the oocyte and it is activated only at the end of vitellogenesis. The binding between the progesterone and the receptor activates the two enzymes CDC2 and cyclin B, which in turn will lead to the synthesis of the maturation promoting factor (MPF). This factor favors an asymmetrical division: the nucleus migrates to the periphery, under the membrane, thanks to the coalescence of yolk proteins and lipid droplets (Carnevali et al., 2020). At the end of maturation, the oocyte is ovulated. After the ovulation, empty

follicles and oocytes that failed the maturation undergo a process named follicular atresia (Lubzens et al., 2010). This is an apoptosis process that allows the recovery of non-utilizing components. The first cells involved in the apoptosis are the granulosa cells. These cells send death signals to the oocyte which recovers all the components that can then be used as a source of energy by the other oocytes through the autophagy mechanism (another process of "clean death"). Once all the recoverable components are retrived, the oocytes goes into apoptosis. An atretic follicle is characterized by hypertrophy of the theca and granulosa cells, degradation of the zona pellucida and vacuolization (vesicles with lysosomal enzymes) of the oocytes (Carnevali et al., 2020). Atresia is a physiological process, but if it overcomes the production processes it represents a sign of a pathological state. This pathological condition could appear due to stress conditions, the presence of pollutants or when energy reserves are not sufficient.

During the reproductive cycle, all these events of oocyte maturation are reflected in an enlargement of the gonad, which changes considerably in size. This allows us to macroscopically define whether the gonad is in the reproductive phase or spent, but not if the organism is mature or immature.

1.2.3 Spermatogenesis

Spermatogenesis is a process through which spermatogonia (male germ cells) differentiate into spermatozoa and it takes place in the testis.

The structure of fish testis varies according to the specie. In teleosts they are two equal elongated organs and consist of two compartments, one germinal and one interstitial, separated by a basement membrane (Schulz et al., 2010). When the testes are mature, they occupy the entire abdominal cavity. Based on the morphology of the germinal compartments, the following types are found:

- lobular, the germinal compartment is organized in seminiferous tubules that terminate blindly at the periphery, towards the vas deferens;

- tubular, the germinal compartment does not end at the periphery, but is highly branched (Bobe, 2010).

The lobular testis, based on the distribution of spermatogonia, can be divided into two types, limited, which means that the spermatogonia are found only in the periphery and non-limited, where the spermatogonia are randomly distributed throughout the lobule (Bobe, 2010).

Spermatogenesis can be divided into three main phases (Figure 7):

- the multiplication phase, type A spermatogonia differentiate into type B spermatogonia, which in turn differentiate into primary spermatocytes;

- the meiotic division phase, when the primary spermatocytes undergo the first meiotic division;

- the phase of transformation of spermatids into spermatozoa that is called spermiogenesis. It is characterized by morphological changes, that is the formation of the head, neck and tail, typical of the spermatozoa (Andreuccetti et al., 2010).
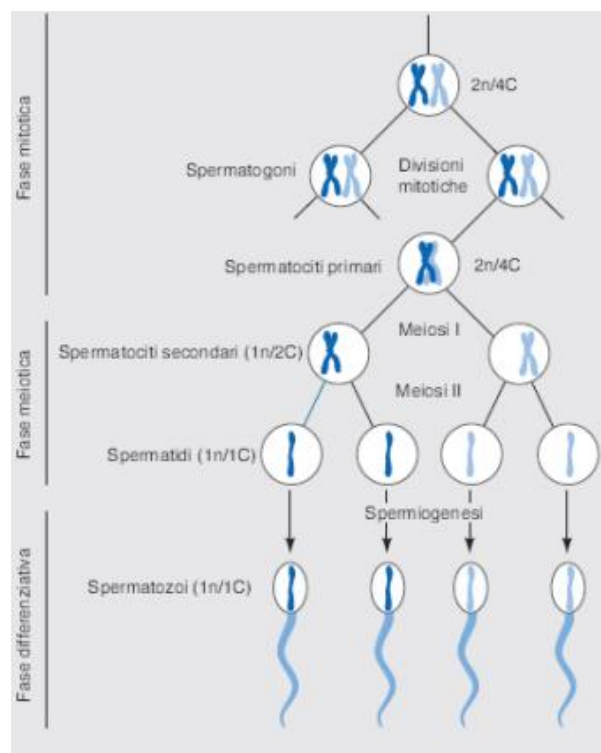

Figure 7. Scheme of spermatogenesis in fishes (Andreuccetti et al., 2010).

During spermatogenesis, the germ cell is characterized by large dimensions which gradually decrease. Germ cells develop in clusters, called germ cysts or spermatocysts (Bobe, 2010).

Also, the spermatogenesis is regulated by the hormones LH and FSH. In teleosts, the receptors for these two hormones are found on the Leydig cells, while the Sertoli cells are mainly regulated by the action of FSH (Schulz et al., 2010). These two hormones stimulate the production of steroid hormones which are the same in both females and males, but they are produced in different quantities. These steroid hormones are:

- androgens, such as testosterone and 11-ketotestosterone;

- estrogens, such as estradiol;

- progesterone.

In teleosts, the male sex hormone par excellence is 11-ketotestosterone, produced by the Leydig cells which exert its function on the Sertoli cells (Schulz et al., 2010).

1.2.4 Reproduction of Sardina pilchardus

The European sardine is a multiple batch spawner, in which groups of oocytes mature at different rates and the females release batches of eggs at different times during the reproductive season, with indeterminate fecundity (Roy et al., 1989). Fertilization is external (males and females release the gametes synchronously, so that the pelagic eggs are immediately fertilized) and there is no parental care. Each female produces from 11337 to 12667 eggs in the peak of its breeding season (Sinovčić, 1984). In the Mediterranean Sea, the sardine reproduces in winter/autumn, in particular in the Adriatic Sea, the reproductive season starts at the beginning of October to April (Morello & Arneri, 2009; Mustać & Sinovčić, 2009; Pešić et al., 2010; Zorica et al., 2016, 2017). The peak of sardine spawning occurs between November and February (Sinovčić et al., 2008; Pešić et al., 2010; Pešić, 2011; Zorica et al., 2017; 2019). The European sardine seems to be a capital breeder (c.b.) (stored energy prior to the spawning season). In Adriatic, the sardine store energy during the period spring/summer and use it for the reproduction in autumn/winter (Ganias et al., 2007; Ganias, 2009; Mustać and Sinovčić, 2009; Pethybridge et al., 2014). As for the first age of sexual maturity, the various data present in the literature suggests that it is around the end of the first year of age (Sinovčić, 1984; Sinovčić et al., 2003). However, the size and

age of early sexual maturity depend on several factors, such as latitude and density-dependent factors (Morello & Arneri, 2009). Also the reproduction is influenced by different biotic and abiotic factors like food availability, temperature (11-16˚C) and salinity (Morello & Arneri, 2009).

Sardines exhibit a migratory behavior linked to the reproductive season. In fact, in autumn/winter the adult sardines move towards deeper and colder waters, while in spring they return to coastal waters (Mužinić, 1973) (Figure 8). Adult sardines migrate offshore, towards deeper and colder waters to reproduce (Mužinić 1973). However, at the beginning of the spring season, adults, larvae and post-larvae return to coastal waters (Mužinić 1973) that are richer in nutrients, with lower salinity and warmer. There are therefore two migratory cycles: one in late summer and the other in early spring (Mužinić 1973).
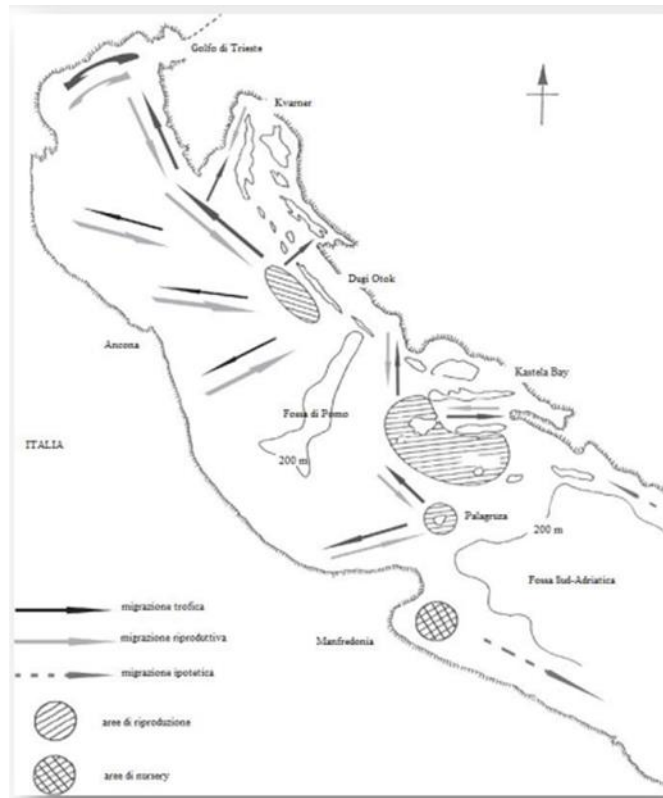
Figure 8. Trophic and reproductive migrations of *Sardina pilchardus* in the Adriatic Sea and representation of nursery area (Morello & Arneri, 2009).

Two reproduction areas have been detected in the Adriatic Sea separated to each other by the Jabuka pit (Piccinetti et al., 1981; Regner et al., 1981, 1984, 1987; Gamulin & Hure, 1983). One is close to Dugi Otok island, at north of the Jabuka well, while the other one is located in the south, around the Dalmatian islands and it extends to the coast of Palagruža. When the total biomass of sardines increases during the breeding season (Casavola et al., 1985), these two areas can also merge and the south reproduction area can extend to the Italian coast, below Otranto (Piccinetti et al., 1981). In 2018, Sciascia and collaborators identified about 9 nursery areas in the Adriatic Sea,

as illustrated in figure 9 (Piccinetti et al., 1981; Regner et al., 1981, 1987; Sinovčić, 2001, 2003; Sinovčić e Alegriahernandez, 1997).
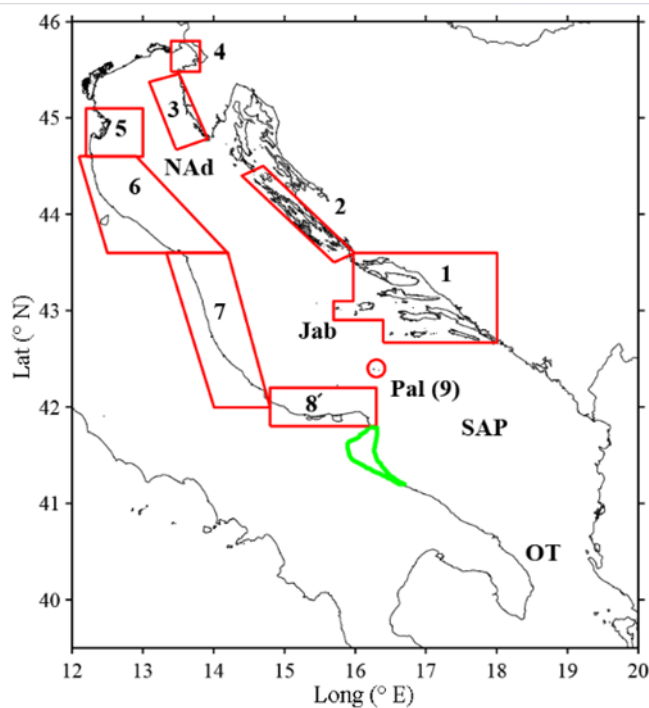

Figure 9. Representation of the nine nursery areas of *Sardina pilchardus* in the Adriatic Sea: the islands of southern Dalmatia (1), the islands of northern Dalmatia (2), the Istrian peninsula (3), the Gulf of Trieste (4), Po River delta (5), coast of northern Italy (6), coast of central Italy (7), promontory of northern Gargano (8) and Palagruža islands (9). (Sciascia et al., 2018).

During ontogenesis, the sardine undergoes a series of morphological and functional changes. Under optimal conditions, the larvae develop rapidly and reach the juvenile stage with a process called metamorphosis, through which they acquire the morphological and physiological characteristics of adults. The transition from one stage to another can be influenced by different factors, such as temperature and nutrition. High temperatures not only lead to

a faster larval grow but can induce ontogenic development earlier and at a smaller size (Garrido et al., 2016; Sciascia et al., 2018).

1.3. Fishing

The sardine is an important biological resource, especially for the fishing and sector of canning industry in several states of the Atlantic and Mediterranean coasts. Two stocks are considered in the Atlantic waters of the EU: the northern one, which are fished by France and Spain fleets mainly, and the southern one, fished by Spain and Portugal fleets (Silva et. al., 2015). While landings of the northern stock have increased over the time reaching the 45,000 tonnes in 2014, the southern stock showed a decrease since the 1980s (Silva et al., 2015).

The French fleet mainly uses two types of fishing gear: purse seines and pelagic trawls. Purse seiners operate in coastal areas, at less than 10 nautical miles from the coast, trawlers operate up to 50 nautical miles offshore. Sardines are one of the most important species for French fishing, representing the 95% of landings (Silva et al., 2015). In the UK, there is also a small traditional small-scale fishing that catches sardines during the summer around the Cornish peninsula. Fishing vessels operate with drift and ring nets (Parkes, 2010).

As already mentioned, the southern stock is mostly fished by Spain and Portugal fleets and, both the countries, fish using purse seines. Due to the

stock decrease in the '80s, regulations have been adopted since 2011 resulting in a 32% decrease of sardines landings from 2011 to 2012 and 39% between 2013 and 2014. In this southern country, there are also two types of artisanal fishing that have a strong social and cultural importance: the "xeito" in Galicia, working with driftnets to catch adult sardines, and the "sardinheira" which is a small fishing using driftnets. The latter operates in the summer along the coasts of Lisbon (Silva et al., 2015).

Concerning the Mediterranean area, sardines and anchovies are the two most important commercial species in the Adriatic Sea, representing approximately 41% of the total catch (FAO 2007). These two species represent a single stock exploited by several countries such as Italy, Slovenia, Croatia, Albania and Serbia-Montenegro. Nonetheless, the eastern and western coasts along the Adriatic Sea show a different preference between the two species, the Italian fleet focuses mainly on anchovies (considered the most commercially relevant), while the fleets of the eastern Adriatic focus on sardines. Total sardine catches, in the Adriatic Sea, have been subjected to various fluctuations, ranging from around 89,000 tons in 1981, to 21,000 tons in 2005 and 79,000 tons in 2016 (SAC-GFCM, 2016) (Figure 10).
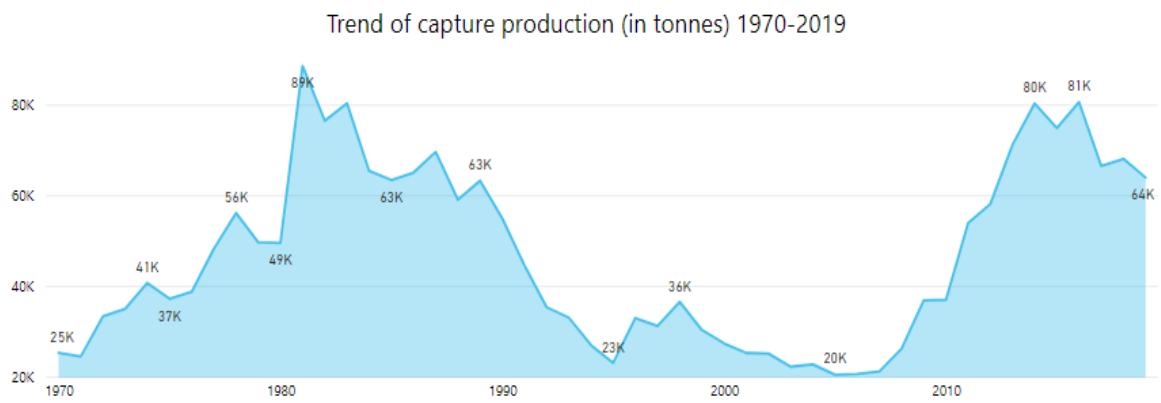
Figure 10. Total catches of sardine in the Adriatic Sea (FAO) (www.fao.org).

1.3.1 Western Adriatic

In the western Adriatic, the maximum amount of sardines caught dates back to 1981 with around 57,000 tons. This peak was followed by a decrease in the stock across the Adriatic Sea as a result of overfishing. For the Italian Adriatic fleet, this decrease reached a value of approximately 3000 tons in 2006.

The fleets involved in the fishing of small pelagics are distributed from Trieste to Bari, the most productive operates between Trieste and Vieste (Cingolani et al. 1996, Santojanni et al. 2001, Cingolani et al. 2003). The Italian fleet is composed by 50 pairs of *volanti* and 40 *lampare*, both types of fishing exploit the gregarious behavior of small pelagics. *Volanti* operate with two boats tha pull a semi-pelagic net and are mainly used in the central-northern Adriatic (Figure 11A). This type of fishing is carried out during the day, when sardines and anchovies move towards the surface for foraging, forming large schools (even multispecific ones). The *volante* operates at about 10-25 miles from the coast and is daily. This means that the boats leave in the morning (around 03:00/ 05:00) and return in the afternoon (around 16:00/ 18:00), for sale at the market. Furthermore, this type of fishing can reach a catch of 2000 boxes per day per pair of boats. *Lampara* (called also saccaleve or purse seines), on the other hand, is mainly used in the south of Ancona,

even if a part operates in the Gulf of Trieste (Falco et al. 2007). It operates on quiet nights, except when there is a full moon, at about 30 to 40 miles from the coast. These boats have lights that attract the plankton which rise, followed by schools of fish which are then captured with purse seines (Figure 11B). *Lampara* set sail in the late afternoon, (around 17:00/ 19:00, or evening, around 22:00) while the return takes place in the morning after (between 07:00/ 09:00). This type of fishing requires good weather and sea conditions, therefore the fishing days per year are less than the fishing with the *volante*.
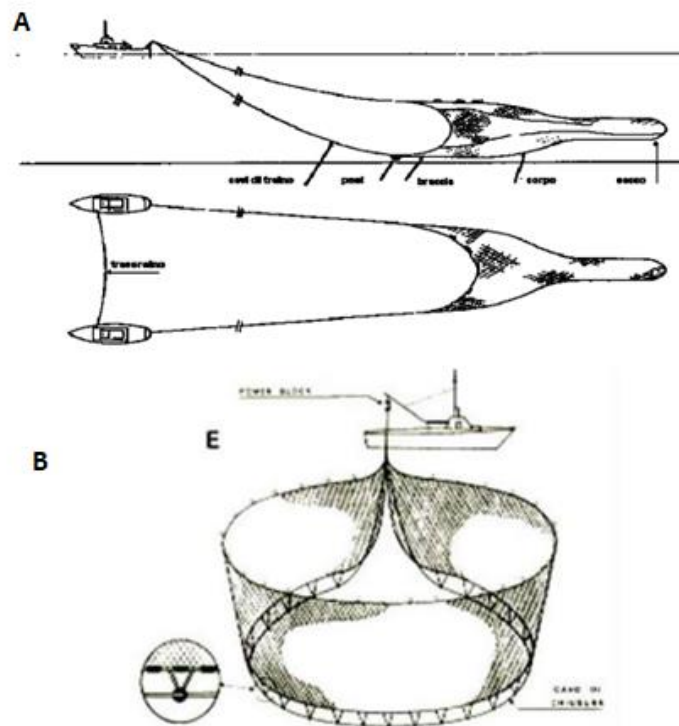

Figure 11. Fishing practice with the *volante* (A) and with the *lampara* (B).

The main ports are Chioggia, Porto Garibaldi, Cesenatico, Cattolica, Ancona, San Benedetto del Tronto and Vieste, but also other ports such as Caorle, Goro, Rimini, Fano, Giulianova and Ortona are of some importance (Cingolani et al., 1996; Santojanni et al., 2001; Cingolani et al., 2003). In the port of Ancona operate four pairs of *volanti* (8 boats), which fish for about 12 hours both in the south and north of Ancona. The fishing is multispecific, (i.e. they catch both sardines and anchovies), and lasts all year round. The fishing rests take place in June, during the reproduction period of anchovies (*Engraulis encrasicolus* (Linnaeus, 1758)), and in October, for the reproduction of sardines (*Sardina pilchardus* (Walbaum, 1792)).

1.3.2 Eastern Adriatic

In the eastern Adriatic, fishing mainly focuses on sardines and takes place mainly with purse seines (*lampara*). Croatia has the largest catches of sardine with a maximum of 16,000 tons in 2005 (Figure 12A). Croatian fleets are distributed along the coast, from Umag to Dubrovnik, fishing mainly between Istria and the Dalmatian islands (Škrivanić and Zavodnik 1973; Tičina et al. 2000; Cingolani et al. 2003). In Slovenia the quantities landed are decidedly lower than the previous ones; they reduced from 3000 tons in 1992 to 1 ton in 2019 (FAO) (Figure 12B).
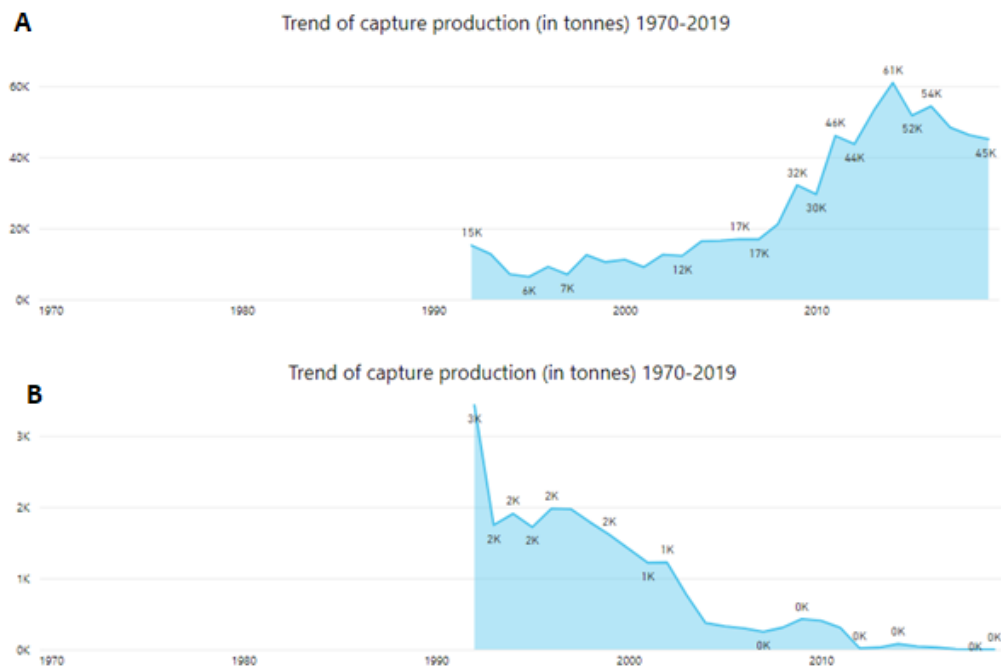

Figure 12. Trend of capture sardines of the coast of Croatia (A) and Slovenia (B) (FAO) (www.fao.org).

1.3.3 Bianchetto

A traditional fishing activity, practiced for several centuries in Italy, is that of the "bianchetto", or the larvae (25-35 mm long) or the young of the Clupeidae and Engraulidae specimens (Figure 13). Larvae up to 40 mm in length have a transparent and depigmented appearance, when they reach greater size they become pigmented.
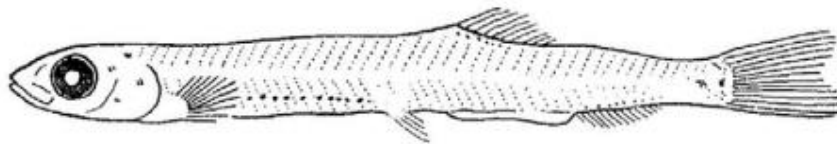
Figure 13. Larvae of sardine, "Bianchetto" (Tortonese, 1970).

The "Bianchetto" fishing is very important for small Italian fishing, as the product has a rather high commercial value. For these reasons this product is also called "white gold". Over time this type of fishing has experinced:

-a decrease in adult individuals and larger sizes, thus increasing the smaller sizes;

-a reduction of the spawners.

For this reason, the "bianchetto" fishery was then regulated to guarantee not only the protection of the environment but expecially the maintenance of the

stocks within safe biological limits. To date, in fact, it is practiced only in areas where an adequate management plan is in force. Furthermore, with the authorization of the Ministry, it is carried out in limited periods of the year, ie in the two months between January and March, when the catches are scarcer due to adverse weather and sea conditions (Romanelli et al., 1998). The fishing tool is the "seine" (or also "sciabegotto" or "bianchettara"), that is a long trawl net with very fine sack meshes (opening 3 mm) to prevent individuals from escaping. This net also has a cork file at the top and a lead file at the bottom to keep it open. The boats, usually with a crew of only two men, lower the net in a semicircular way, at this point some men on the beach pull the net. In some areas, such as in Linguria, the net, after having surrounded the school of fish, is retrieved directly on board or by means of rollers or by arm. This type of fishing can take place both during day and night, through the use of a light source that attracts the school of fish.

1.4. Age and growth

The study of age is very important to establish the rate of growth, mortality and productivity of species (Campana, 2001). Over the years, several methods of determining the age have been developed: at first the vertebrae were used (Henderstrom, 1959), then the scales (Carlander, 1987) and finally the otoliths (Ricker, 1975).

Otoliths (from the greek *oto*= ear and *lithos*= stone) are concretions of calcium oxalate which are deposited at the level of the vestibular apparatus and have a state-acoustic function. They are located within the semicircular canals of the inner ear and provide information to the nervous system about changes in movements. In fishes, the back of the cranium presents the inner ear, a complex structure consisting of channels, sacs and ducts. Teleosts have three semicircular canals arranged orthogonally to each other and they open inside large chambers or otic sacs containing the macula (a sensory tissue that senses sound waves and acceleration) (Panfili et al., 2002) (Figure 14). There are three types of otic sacs: *utricle*, *saccule* and *lagena* and each of these contains a pair of otoliths, respectively *lapillus*, *sagittae* and *asteriscus*.
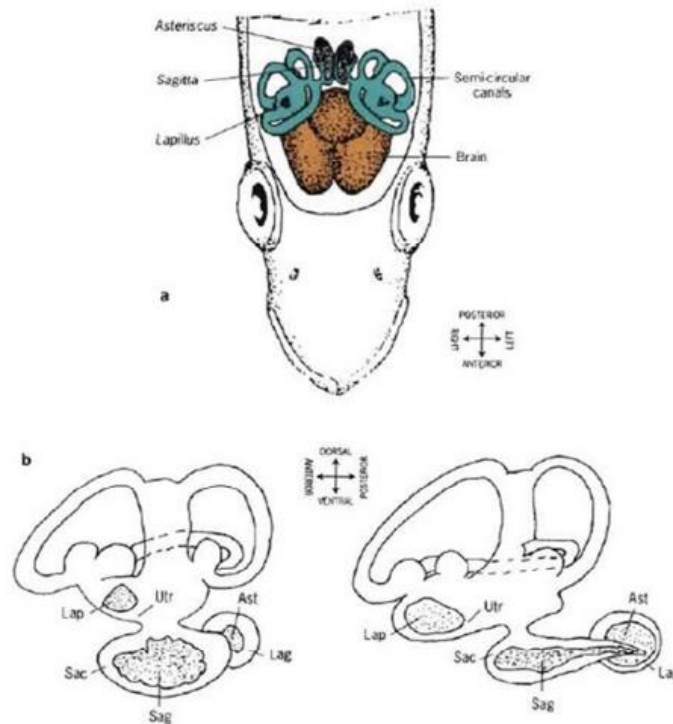
Figure 14. Localization of otoliths in the inner ear of the Teleost. (A) Dorsal view of the vestibular apparatus in a typical Teleostei species. (B) Otoliths within the labyrinth system of typical Teleost and Ostariophysean fishes (Secor et al., 1992).

The *sagittae* have a greater morphological variability compared to *lapilli* and *asteriscus,* for this reason they are most used for age determination (Assis, 2005). The *sagittae* are characterized by a convex zone (dorsal) and a concave one (ventral). The concave side has the acoustic sulcus, which communicates with the sensory epithelium (the macula) and presents nerve fibers coming from the sides of the *sacculo* (Panfili et al., 2002). Furthermore, at the front they have an elongated structure, called *rostrum*, which lengthens in the course of ontogenesis.

Otoliths differ in function, size, shape and microstructure (Secor et al., 1992). The morphometry of the otoliths is useful for the taxonomy of Teleosteis (Hecht, 1979) and reflects the influence of environmental changes in the habitat (Morales-Nin, 1987). Otoliths show a range of incremental structures, called rings or bands, formed by the alternate deposition of calcium carbonate and otoline (a protein matrix) (Secor et al., 1992). It is through these bands that it is possible to determine the age. In fish, moreover, this determination can take place through two time scales: daily and annual (Campana, 2001). Daily scale is applied mostly to study of juvenile fish (Pannella, 1971; Campana & Neilson, 1985). Pannella in 1971 discovered that in juvenile organisms, i.e. those that have not yet turned one year old, it is possible to determine age by analyzing finer rings located immediately near the core (Jones, 1992). The annual scale, on the other hand, is used for to study the population and presents the seasonal increases, also called *annuli*. These *annuli* are composed of an opaque band and a hyaline band (the different bands reflect the change in temperature and therefore seasonality). Furthermore*, annuli* are easily recognizable in tropical and temperate species (Panfili et al., 2002). In several species, however, the study of annual increases can be difficult because of the presence of the so-called false rings. These false rings appear as translucent areas within the opaque areas and

could lead to an incorrect age determination. Although the causes are still poorly understood, it is believed that factors such as temperature, food consumption and developmental stages may be responsible for the formation of the false rings (Panfili et al., 2002) (Figure 15).
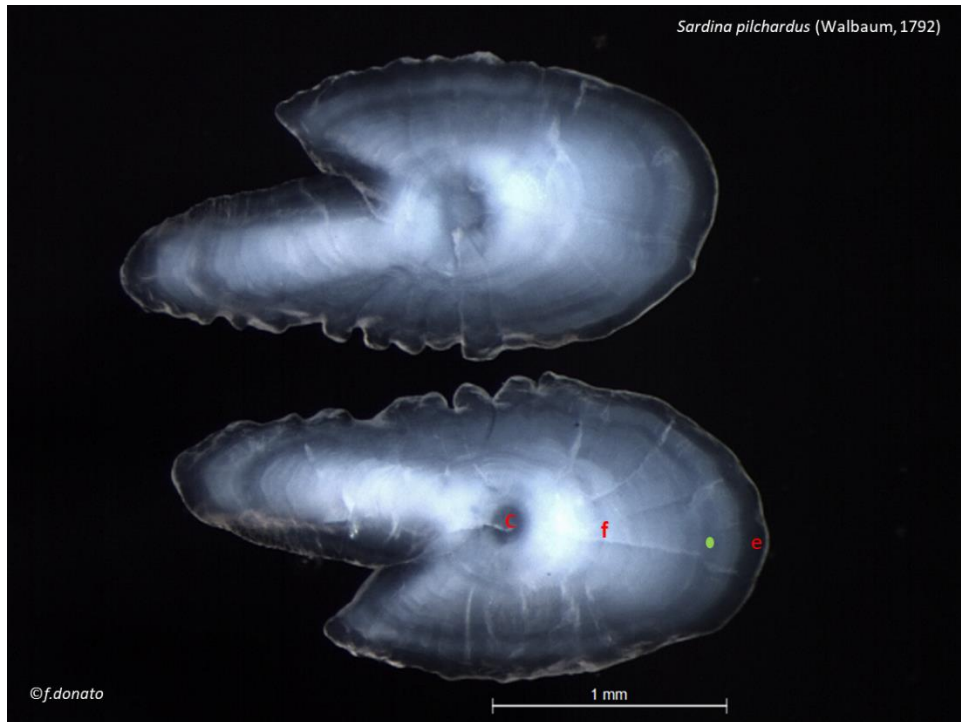

Figure 15. Otoliths (*sagittae*) of *S. pilchardus*. C= core or nucleus; f= false ring or check; green dots= winter ring; e= hyaline edge.

2. AIM OF STUDY

Sardina pilchardus (Walbaum, 1792) is one of the most important fish resources in the Adriatic Sea of both ecologically and socio-economically relevance. As the other small pelagic species, sardines play a key role in the ecosystem since they are able to exert both bottom-up and top-down control type, by regulating both the abundances of high trophic level (carnivorous fish, seabirds and marine mammals) and the plankton species. Moreover, they represent the majority of landings deriving from both large-scale (industrial) and small-scale (artisanal) fisheries.

The overall purpose of this thesis is to deepen the knowledge of sardine reproductive cycle in the middle-west Adriatic Sea focusing on female gonads maturation and development correlated with the fishing areas and the age growth.

This study was performed in collaboration with the local fishermen (which provided the biological samples) operating in the waters off the coast of Marche region and consisted in the analysis of samples collected within a period of 13 months. Specifically, the thesis was divided into three parts.

The first aim was to characterize histologically the ovary structures at different reproductive stages. Secondly, the focus was set on determining the

age of both male and female specimens to understand the distribution of age classes within the population. Finally, the information obtained on the reproduction aspects were correlated to all data collection concerning the fishing activity of Ancona's fleet. The results discussed in this thesis were obtained through a multidisciplinary approach which included, biometric, histological and otolith analysis performed within the collaboration among the Polytechnic University of Marche (Ancona, Italy), the CNR's Institute of Marine Sciences of Ancona (Italy) and the University of Catania (Italy).

3. MATERIAL AND METHODS

3.1 Sampling

From March 2021 to March 2022, a monthly sampling was performed (for a total of 1803 individuals) (Figure 16A) in collaboration with the Ancona fleet (made up of 8 *volanti*) operating in GSA 17 (FAO Geographical Area 37, Subarea 37.2) which provided the sardine specimens. Due to the fishing prohibition, no sampling was carried out during the months of June and October 2021. At each sampling time, from the total amount of sardines, 417 specimens were individually weighted and length measured (from the tip of the snout to the margin of the caudal fin) (Figure 16A) before their sorting into different size classes following this criterion: the size class range was of 0.5 cm, each size class (ranging from 11 cm up to 15,9 cm) consists of 3 males and 3 females. Once measured and weighed, the gonads were taken and weighed (Figure 16B-C). For each gonad 3 portions were taken: distal, central and proximal. Too small gonads were taken whole. Finally, the sampled gonads were fixed in formol solution at 4°C for histological analysis. The classified individuals were also stored -20 °C and subsequently transported to the CNR of Ancona for the extraction and analysis of the otoliths.

The remaining specimens, approximately 100 individuals every month, were randomly measured for the biometric parameters (length and weight) and sex determined.
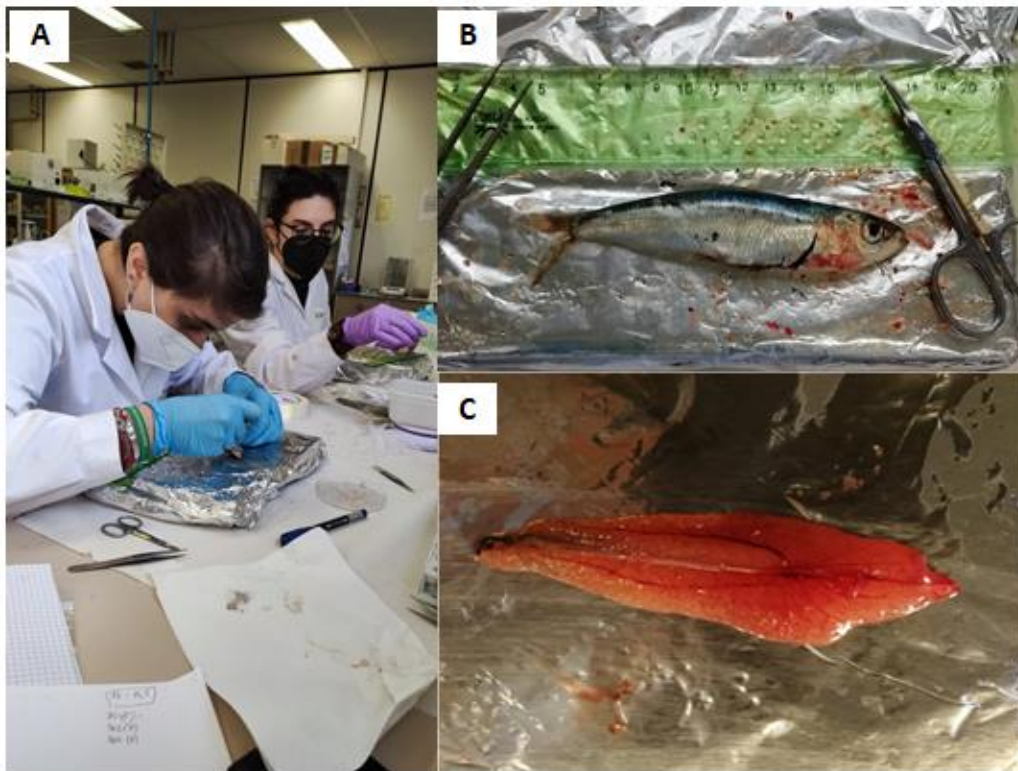

Figure 16. (A) Sampling of sardine; (B) Example of sampled sardine; (C) Female sardines ovary.

Concerning fishing data, ad hoc logbooks (Figure 17) were filled monthly by fishermen and used to register the following information:

- vessel data (name, meters, tons and engine power);

- fishing area and distance from the coast;

- duration of fishing (time of departure and return);

- duration and number of hauls;

- tool;

- target species and quantity caught (in kg);

- by-catches.

Furthermore, additional information was collected on the state of the fishery and the commercial value over the past years. These data were used to: calculate the fishing effort and strength of Ancona's sea fishing; identify fishing areas; evaluate the by-catches and, finally, define the commercial trend of the species.

Figure 17. Logbook example used in this study.

3.2. *GSI and sex ratio*

The gonadosomatic index (GSI), was determined for the sardines sampled using the following formula:

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

where GW is the gonad weight (g), and WT the total weight of the fish (g).

The sex ratio was determined both macroscopically considering the morphological aspect of the gonads and through histological analysis.

3.3. Histological analyses

- Paraffin inclusion

Histological analyses were conducted on 207 female gonads (3 specimens per size class) to establish their reproductive stage and on few male gonads (only of July, August and September) to confirm the sex. After fixation in formol, the samples were washed twice (15 min each) in 70% ethanol. Successively, the samples were placed in biocassettes to be dehydrated through immersion in decreasing ethanol solution (70%, 80%, 95% and 100% ethanol). Samples dehydration is essential to remove excess water and fixative. After this procedure, the protocol provides step in xylene to remove all the ethanol residuals and prepare the samples for paraffin embedding. Finally, samples were included in paraffin (Bio-Optica) and left to solidify overnight at room temperature.

- Microtome and staining protocols

Once the paraffin blocks were solidified, they were cut with a microtome into 5 μm sections. For each sample 3 sections were cut, each at a distance of 20 waste cuts, and placed on a glass slide. The slides were left overnight, at room temperature, so that the sections adhered completely to their surface.

Sample slides were then stained following Mayer haematoxylin and eosin Y (Merck KGaA) protocol.

- Optical microscope

Each section was analysed using an optical microscope (ZEISS Axio Imager M2, Oberkochen, Germany) and images were acquired using a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany). Through the Brown-Peterson criteria (2011) (Figure 18), it was possible to determine the reproductive stage of each analysed femal.

Phase	Previous terminology	Macroscopic and histological features
Immature (never spawned)	Immature, virgin	Small ovaries, often clear, blood vessels indistinct. Only oogonia and PG oocytes present. No atresia or muscle bundles. Thin ovarian wall and little space between oocytes.
Developing (ovaries beginning to develop, but not ready to spawn)	Maturing, early developing, early maturation, mid-maturation, ripening, previtellogenic	Enlarging ovaries, blood vessels becoming more distinct. PG, CA, Vtg1, and Vtg2 oocytes present. No evidence of POFs or Vtg3 oocytes. Some atresia can be present. *Early developing subphase*: PG and CA oocytes only.
Spawning capable (fish are developmentally and physiologically able to spawn in this cycle)	Mature, late developing, late maturation, late ripening, total maturation, gravid, vitellogenic, ripe, partially spent, fully developed, prespawning, running ripe, final OM, spawning, gravid, ovulated	Large ovaries, blood vessels prominent. Individual oocytes visible macroscopically. Vtg3 oocytes present or POFs present in batch spawners. Atresia of vitellogenic and/or hydrated oocytes may be present. Early stages of OM can be present. *Actively spawning subphase*: oocytes undergoing late GVM, GVBD, hydration, or ovulation.
Regressing (cessation of spawning)	Spent, regression, postspawning, recovering	Flaccid ovaries, blood vessels prominent. Atresia (any stage) and POFs present. Some CA and/or vitellogenic (Vtg1, Vtg2) oocytes present.
Regenerating (sexually mature, reproductively inactive)	Resting, regressed, recovering, inactive	Small ovaries, blood vessels reduced but present. Only oogonia and PG oocytes present. Muscle bundles, enlarged blood vessels, thick ovarian wall and/or gamma/delta atresia or old, degenerating POFs may be present.

Figure 18. Macroscopic and microscopic descriptions of the reproductive cycle stages of female fish (Brown-Peterson et al., 2011).

3.4. Criteria for the classification of abnormalities in the female gonads

Abnormalities in female gonads were identified during histological analyzes, each alteration was recognized following specific structural characteristics:

- anomalous coalescence of vitellogenin when observed in small oocytes, with anomalous staining and related to the presence of small vitellogenin vesicles in the ooplasm.

- Indefinite structures within the oocytes appearing as membrane-like formation in the vitellogenic stage.

- Atypical high presence of previtellogenic atretic oocytes.

Based on these abnormalities, a grading was established ranging from 0 (no anomaly presents in the gonad sample) to 3 (all anomalies were present in the gonad).

3.5. Otoliths analysis

The analysis of the otoliths was carried out on 417 individuals of both sexes collected from March 2021 to March 2022, excluding the months of fishing stop (June and October 2021). The first step in the analysis of otoliths concerns their extraction. This is done by making a sagittal cut from the base of the cranium to the mouth opening, with a scalpel. With the help of tweezers, the otoliths present at the base of the brain were extracted. Once extracted, the otoliths were cleaned of organic residues, washed with water and dried with paper, and finally placed in numbered eppendorf (the number corresponded to that of the individual). The second step was the reading of the otoliths, which can take place about a month after the extraction. For reading, the otoliths were dipped in ethanol inside a Petri with a black background. An optical stereomicroscope with binocular system was used, connected to a digitized computer video system (Leica Application Suite 4.3.0.). To be read correctly, otoliths must be positioned with the acoustic groove facing downwards.

For individuals that spawning in winter, as in the case of sardines, 1st January has been conventionally set as the date of birth (William and Bedford, 1974). Since sardines are born in winter, the otoliths had a hyaline nucleus followed

by the first opaque ring. The age was determined by counting the *annuli*, consisting of the opaque and hyaline band that form in a year (Figure 19). Over the years, the otoliths become morphologically larger, while the size of the *annuli* decreases. The age determined on the sardines ranges from 0 (they have not completed the year) up to 3 years. For otoliths that are difficult to read or crystallize, a value of 99 was assigned, which is impossible to determine their age.
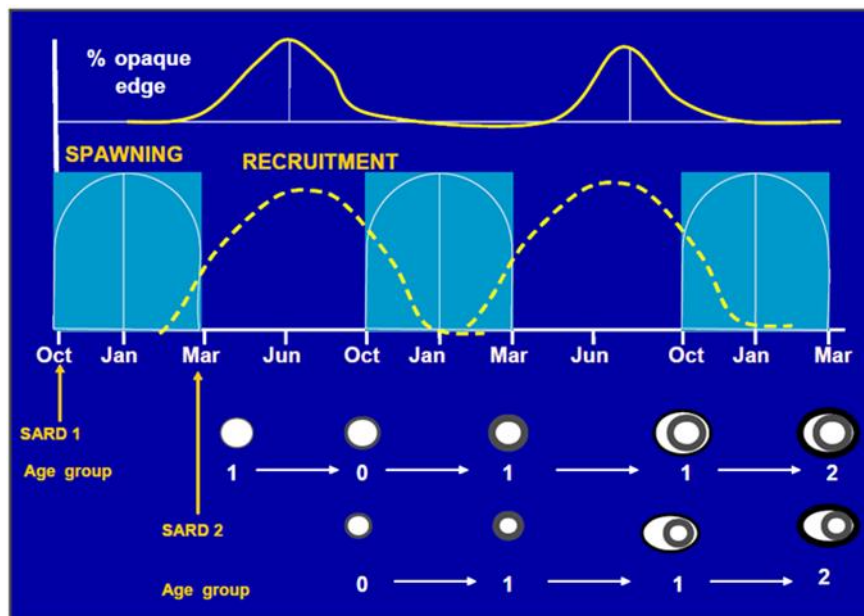

Figure 19. Scheme of the birthdate and otolith margin conventions for sardine age determination.

3.6. Otoliths data

The relationship between the total length to the weight of the fish samples was calculated using this formula:

$$W = aL^b$$

where *a* and b are two constants.

To verify the accuracy of the readings made in determining the age, two statistical indices were calculated: the percentage of average error (APE or Average Percentage Error) and the average coefficient of variation (ACV or Avarage Coefficient of Variation).

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

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:

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

Both sequences were analysed using Excel software.

The coefficient of variation (CV) was calculated with 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 through the formula:

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

The CV formulas was analysed using Excel software.

3.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software for the ANOVA and pairwise Pearson correlation. Significance was set with 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

Sex ratio

Sex of each specimen was determined macroscopically by the observation of the gonads. The sex ratio was calculated considering all sampled specimens, divided according to both the size classes (Figure 20A) and the sampling month (Figure 20B).
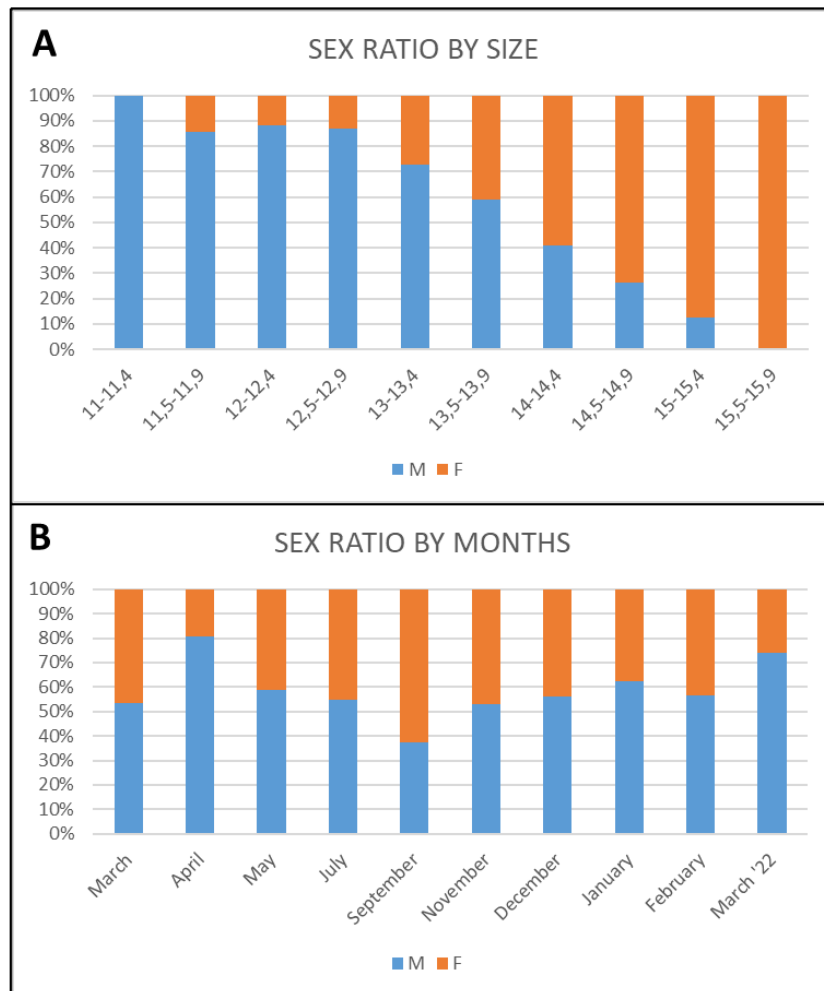

Figure 20. Sex ratio distribution expressed in percentage (%) based on size class (A) and months (B).

As showed in the size-based graph (Figure 20A), there is an opposite trend of females and males' frequency in each group. Males represent the 100% of the smallest size class (11-11.4 cm) while their percentage gradually decrease with the increase of size-class. However, their percentage remains higher than the females one in each class up to the 6th size class (13.5 – 13.9). Conversely, female specimens represent only the 7,69 % in the 2nd class (11.5-11.9) while their number gradually grow in the following classes to reach the 100% of sampled specimens in the highest size class (15.5-15.9). Figure 20B shows the population trend over the months. The total male/female ratio within the whole year is 3:2. During the year samples mainly consist of males, with the highest peaks in April and March 2022 (80.87% and 74.05% respectively). The only exception ca be observed in September when there are more females than males. In Figure 20A and B, data from August are missing. This is related to the maturation stage of the gonads during this month. Since they are out of the breeding season, the gonads are spent and their appearance makes difficult to macroscopically differentiate a testis from an ovary (Figure 21). Therefore, August samples were characterized by the only histological analysis.

Figure 21. Examples of the morphology of ovaries (A) and testis (B) during the reproductive period, and unclassified gonad in the spent period (C).

Macroscopic vs microscopic sex determination

Figure 22 represents the difference between macroscopic and microscopic sexual determination of sardines during the month of September (as an example of spent period) and January (in the middle of the reproductive period). This comparison highlighted that the macroscopic sex determination underrated the males percentage in September, while the same analysis performed on samples from January identified the same number of both male and female specimens obtained through the microscopic evaluation.
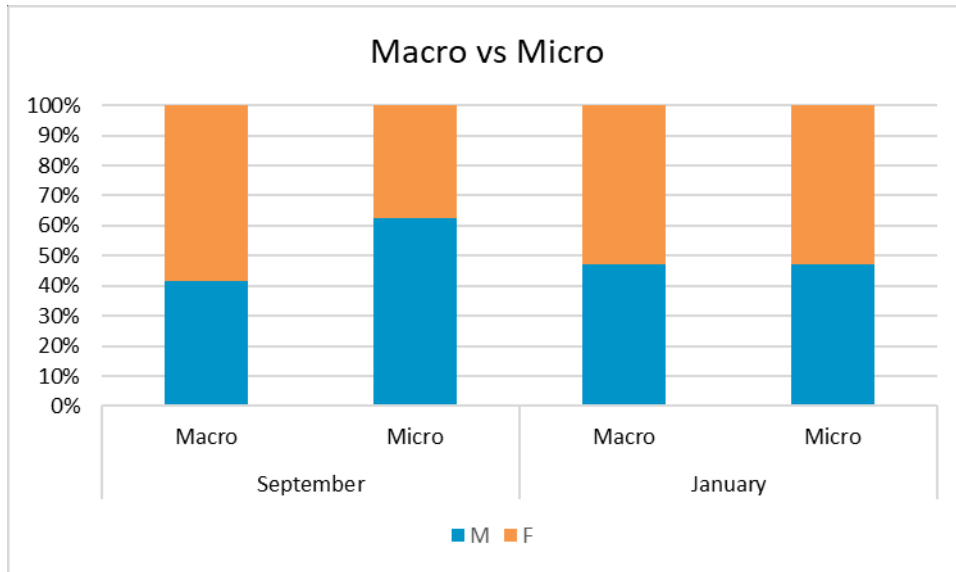
Figure 22. Sex ratio (%) of sardines determined by the macroscopic and microscopic analysis in September and January.

Reproduction stages

The maturation stages of female gonads were identified as follow: developing, spawning capable, and spawning active stages which represent the individual in the reproductive period. While the regressing and the regenerating stages represent mature but not reproducing individuals (Figure 23). This analysis shows the absence of immature females at each sampling time.
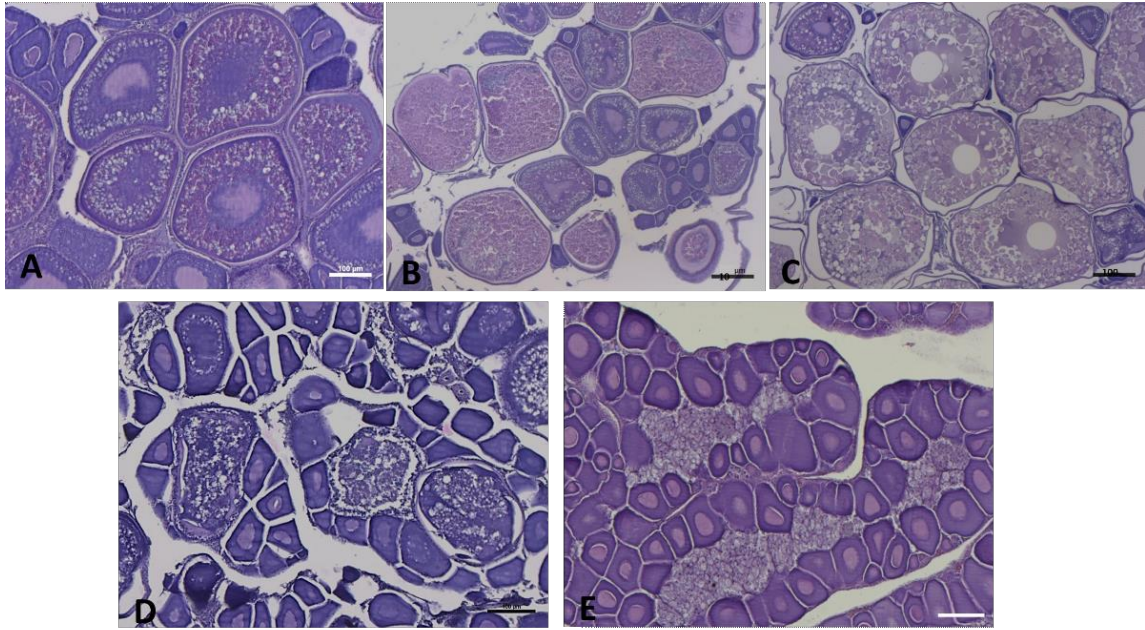
Figure 23. Representative *Sardina pilchardus* ovary images at different reproductive stages determined following the Brown-Peterson criteria: (A) developing, (B) spawning capable, (C) spawning active, (D) regressing (E) regenerating. Scale bars: 100 μm.

Considering the distribution of the reproductive stages among the different size classes (Figure 24A) it can be clearly observed that the developing stage is the only one present in all size classes where it occurs at the highest percentage compare to the other stages. All the other reproductive stages are distributed among the classes between 13 and 15.4 cm, with the exception of spawnig capable stage, observed also in the second and last size class (12-12.4 and 15.5-15.9 respectively). Figure 24B shows that developing stage is also the more rapresentative stage in all age groups, it is presents at 100% in age 3 because this size class is represented just by one female. Spawning capable and regenerating stages occur from age 0 to 2 years old group

following an opposite trend, decreasing and increasing respectively with increasing age. Finally both spawning active and regressing stages were identified in animals belong at 0 and 1 year old groups at similar percentage.
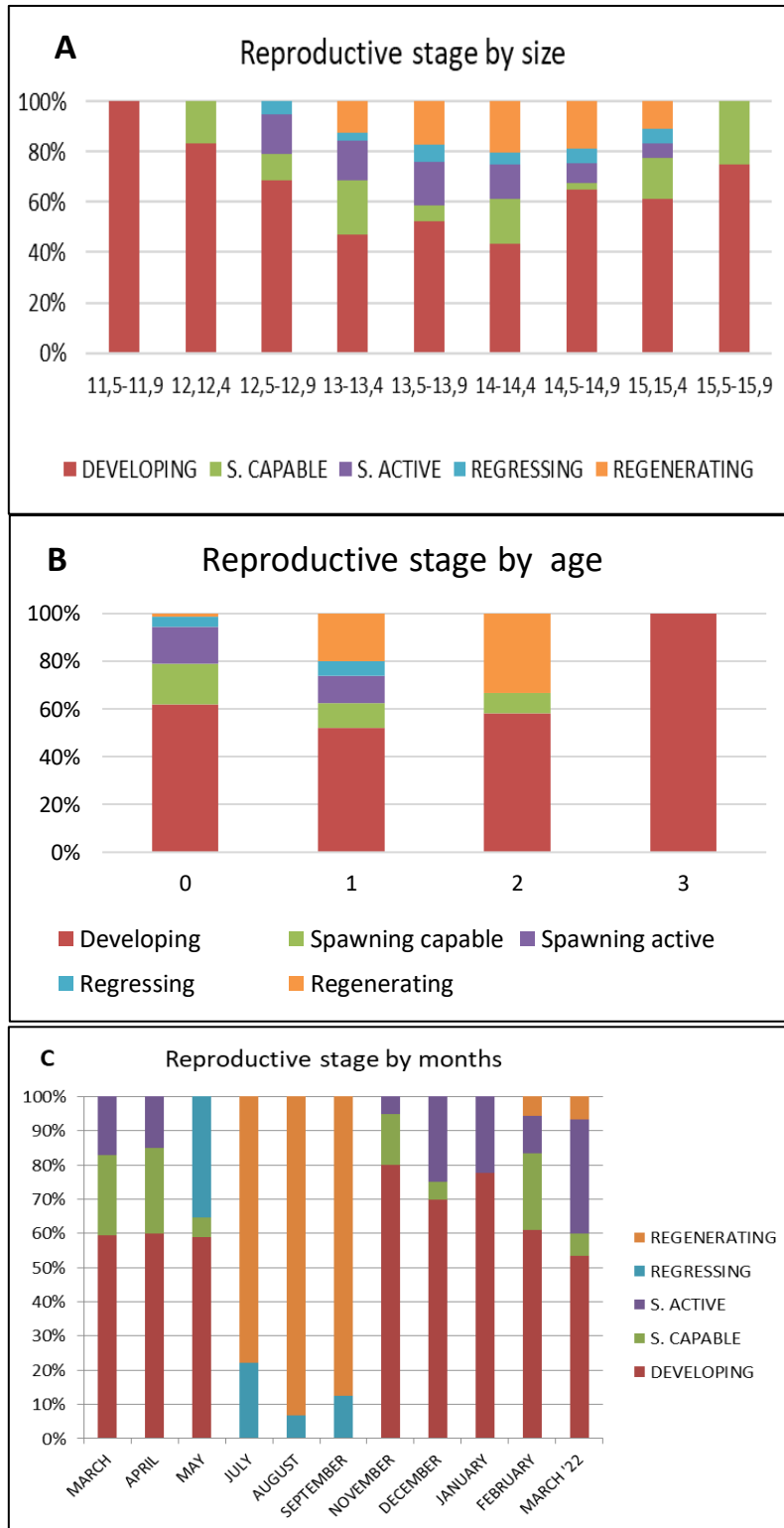
Figure 24. Distribution of reproductive stages over the different months (A), by age (B) and according to size classes (C).

Figure 24C shows the distribution of gonads developmental stages over the sampled months. Developing, spawning capable and spawning active stages are present only from March to May and from November to March 2022. Developing stage is the one with the higher percentage, in all moths in which it is present, respect to spawning capable and spawning active stages which also occur in the same months. July, August and September are reprsented by the regressing and regenerating stages, the last is always at the highest percentage. Also, it can be noted that the 35.29% of females in May are in regressing phase.

GSI

The gonadosomatic index (Figure 25) is represented on a monthly basis, considering only female specimens. The graph shows that the GSI values in the months of the full reproductive period (March, November, December, January, February and March 2022) are significantly higher compared to the values representing the other months (July, August and September).
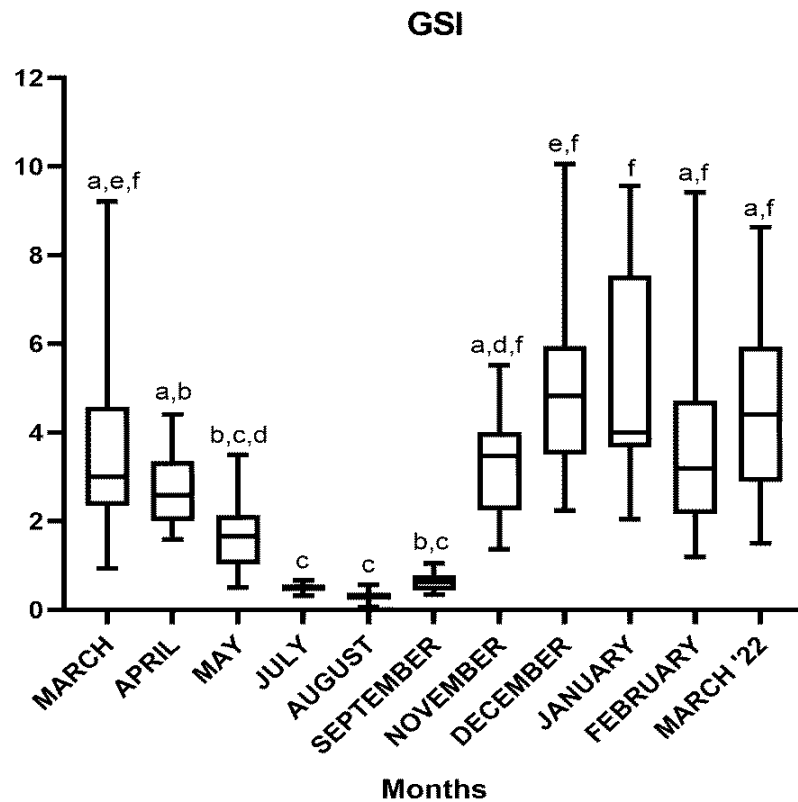
Figure 25. Gonadosomatic index (GSI) values over the months. Significance was set at $p \leq 0.05$. Different letters indicate significant differences.

Abnormalities in the reproductive stages

The figures below show the abnormalities found during the histological analyzes of ovary sections. These abnormalities were classified as: anomalous coalescence of vitellogenin (Figure 26), presence of indefinite structures inside the oocytes (Figure 27) and high presence of previtellogenic oocytes

(Figure 28). A score from 0 to 1 was assigned to each sample based on the criteria described in material and methods section.

Moreover, during the histological samples' examination, other irregular features were observed. Also it was possible to recognize necrosis areas (Figures 29); blood vessels with thickened walls, high number of white blood cells (Figure 30A-B); indefinite parasites (Figure 31) and double oocyte (Figure 32) in a few individuals.
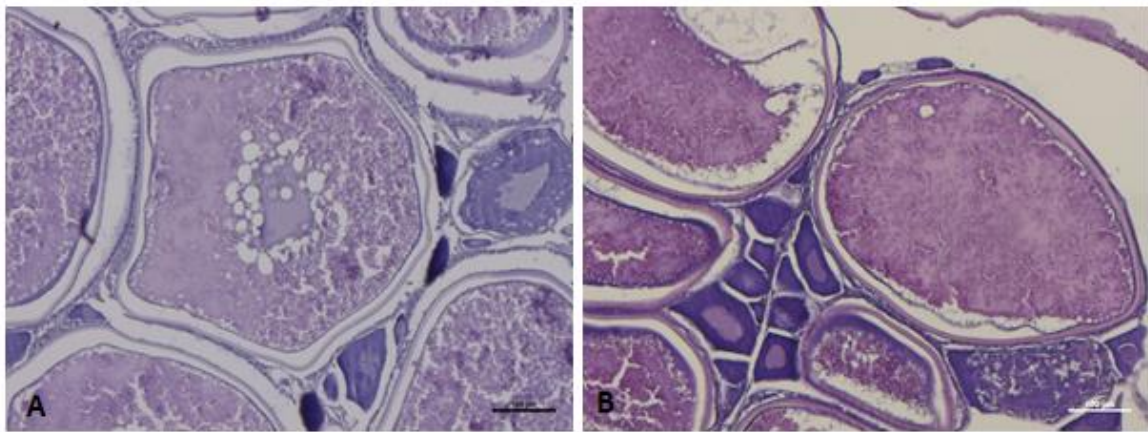
Figure 26. Representative images of sardine ovary with abnormal vitellogenin coalescence in developing phase (A) and in the capable spawning phase (B). Scale bars: 100 µm.
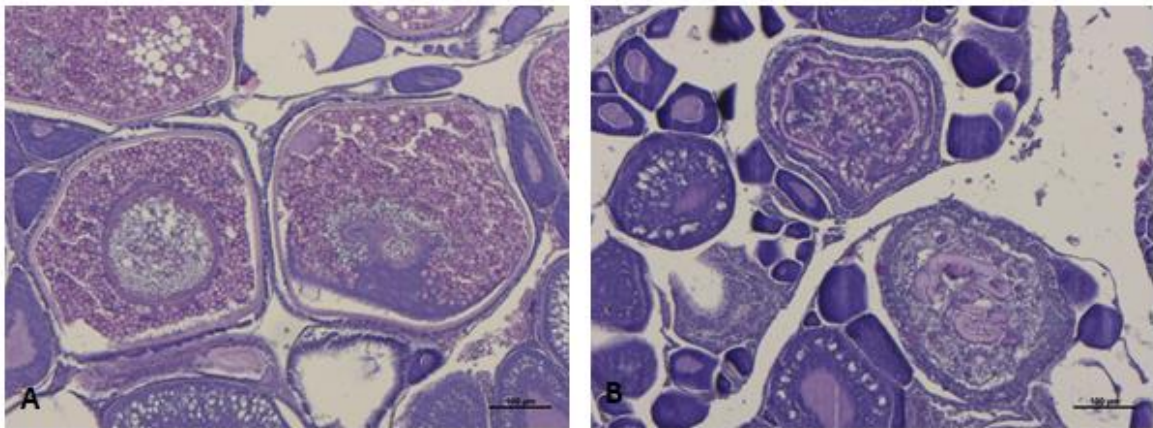
Figure 27. Representative images of sardine oocytes undefined internal structures: (A) in the spawning capable phase; (B) in the developing phase. Scale bars: 100 μm.
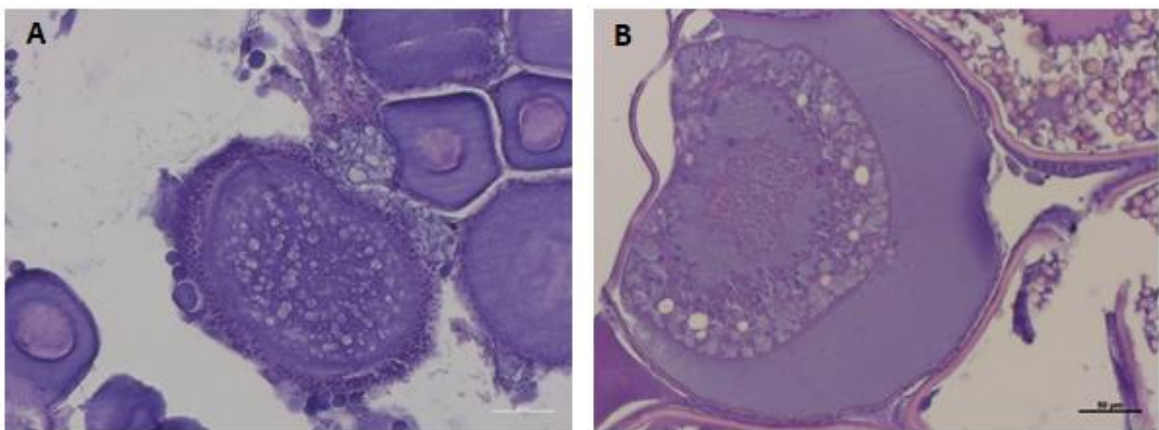
Figure 28. Representative images atretic previtellogenesis oocytes: (A) in the regenerating phase; (B) in developing phase. Scale bars: 50 μm.
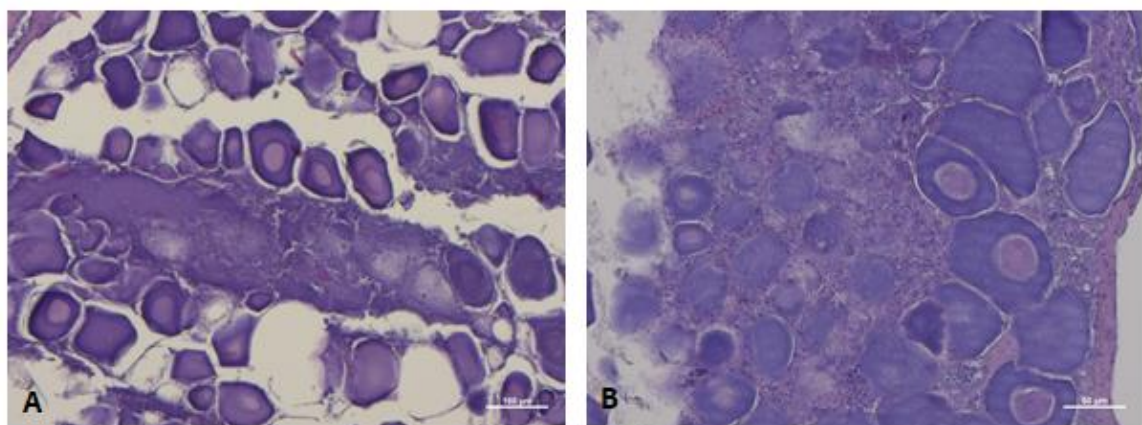
Figure 29. Representative images of necrosis in the female gonad: (A) in regeneration phase, Scale bars: 100 μm; (B) in regenerating phase, Scale bars: 50 μm.
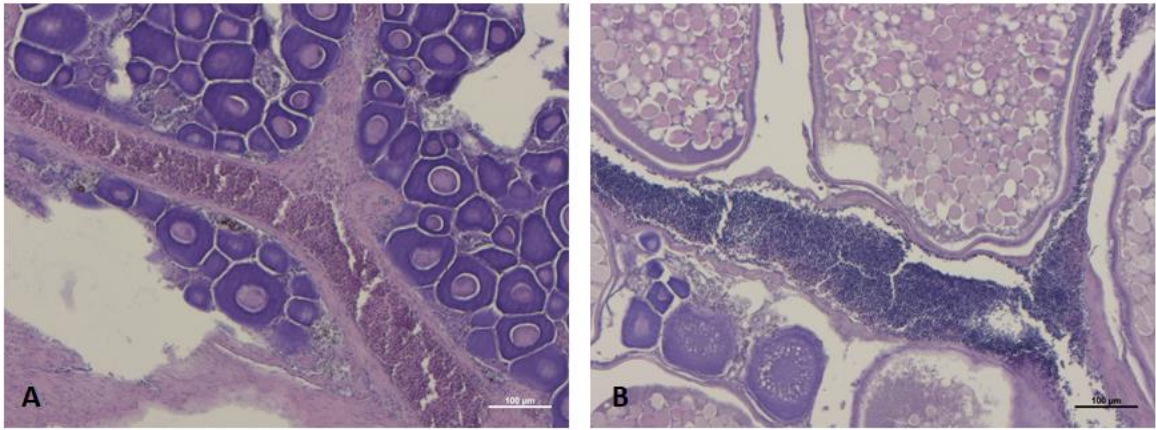
Figure 30. Representative images of blood vessels in female gonads: (A) with thickened wall; (B) with many white blood cells. Scale bars: 100 μm.
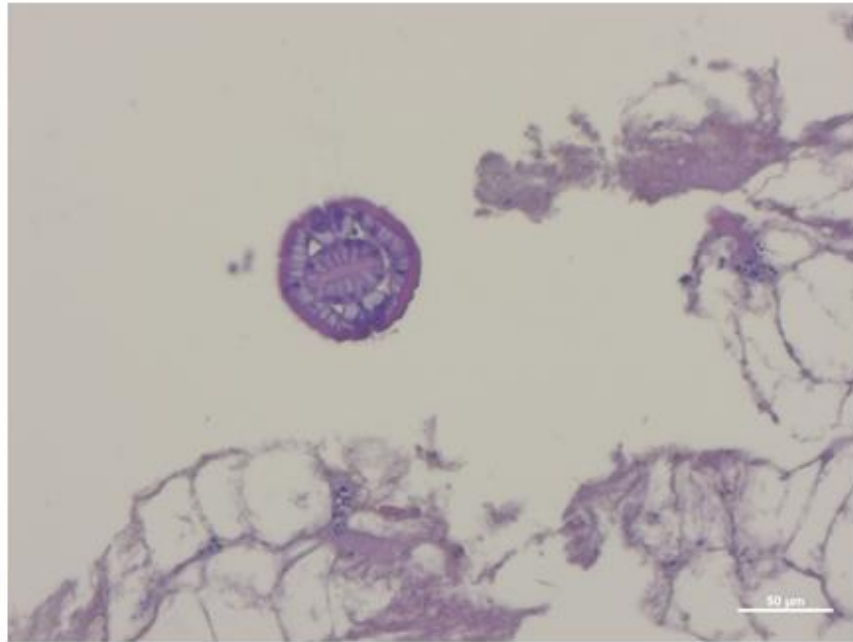
Figure 31. Representative image of an indefinite dissected parasite in the female gonad. Scale bars: 50 μm.
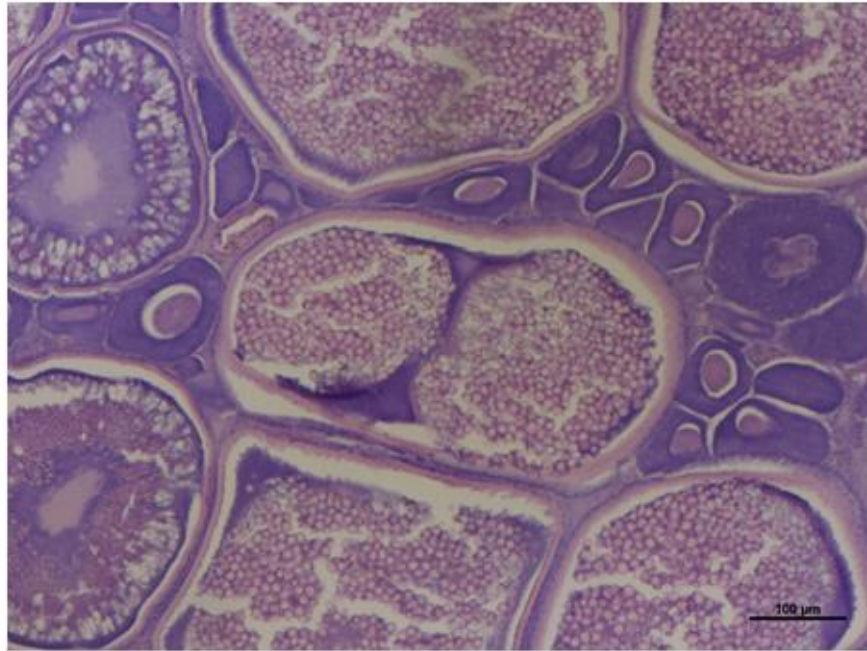
Figure 32. Representative image of a double oocyte, in the developing phase. Scale bars: 100 μm.

As regards the three abnormalities to which a score was entrusted, their occurrence was assessed based on the reproductive stage (Figure 33). The most affected stages are the developing and spawning capable ones. The following graphs highlight the trend of score in the two most affected stages (developing and spawning capable) both among age groups and size classes.
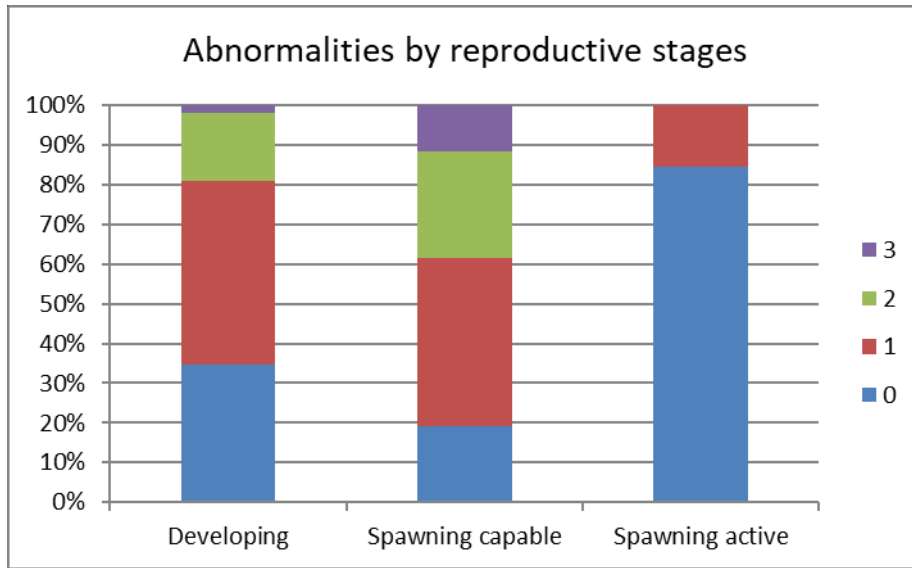
Figure 33. Histogram showing the different abnormalities score assigned to samples at developing, spawning capable and spawning active stages.

Figure 34 highlights the score trend according to the size class, both for developing (34A) and for spawning capable (34B) stages. There is no evidence of anomalies score trends among size classes regarding both reproductive stages considered.
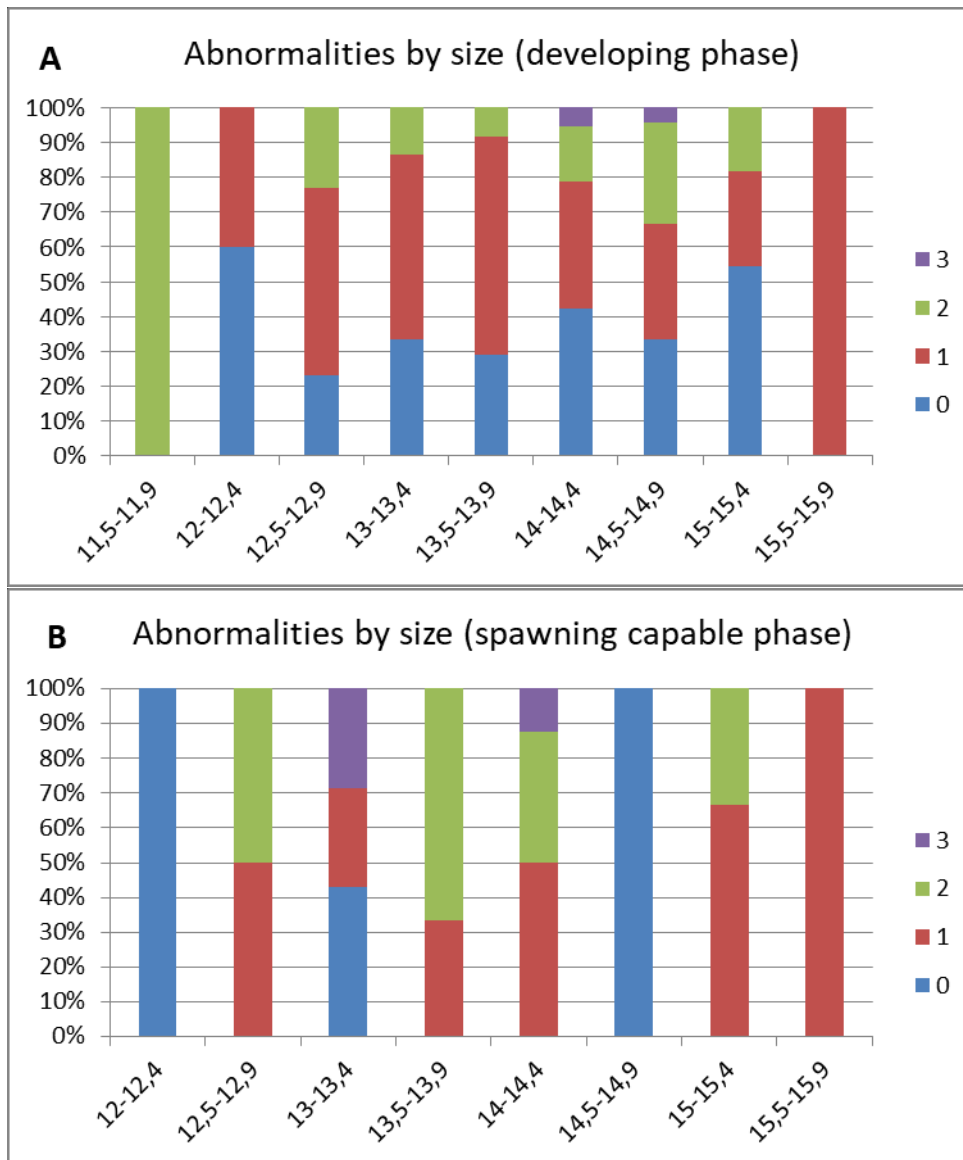
Figure 34. Graphs of the score trend based on the size class, in the developing (A) and spawning capable (B) stages.

The graphs in figure 35 show the trend of the classification based on age, in developing and spawning capable stages. In figure 35A it can be observed that, in the developing phase, healthy females decrease with aging and there is an increase of score 2. On the other hand, in spawning capable phase (Figure

35B) it is observed that the score 3 is already present at age 0 and, as for developing phase, the presence of healthy females decrease with aging.
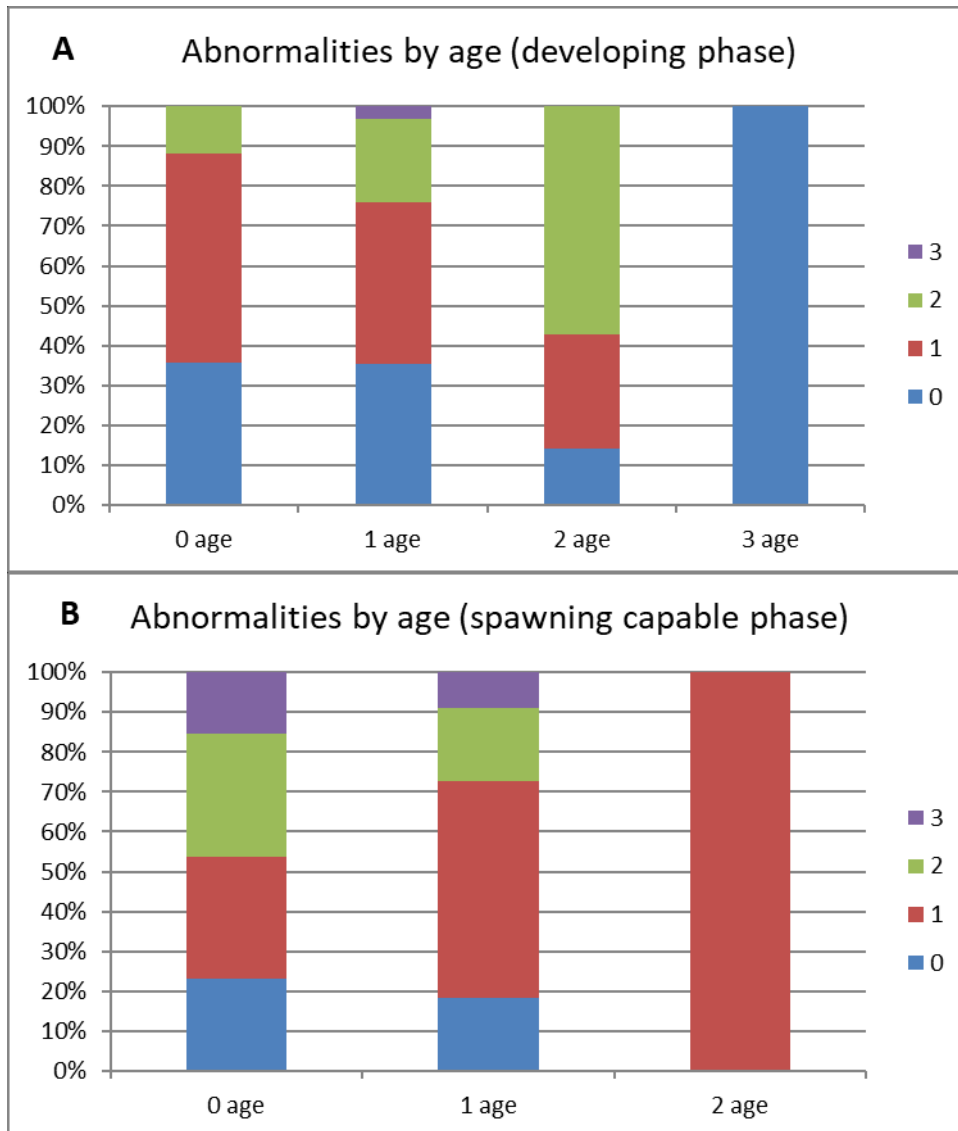
Figure 35. Score trend based on age: (A) in the developing stage, (B) in the spawning capable stage.

Some alterations were found in the few male gonads here analyzed. These abnormalities were: hemolysis (Figure 36A), necrosis (Figure 36B), thickening of the blood vessel walls (Figure 36C) and parasites (Figure 3 D-E).
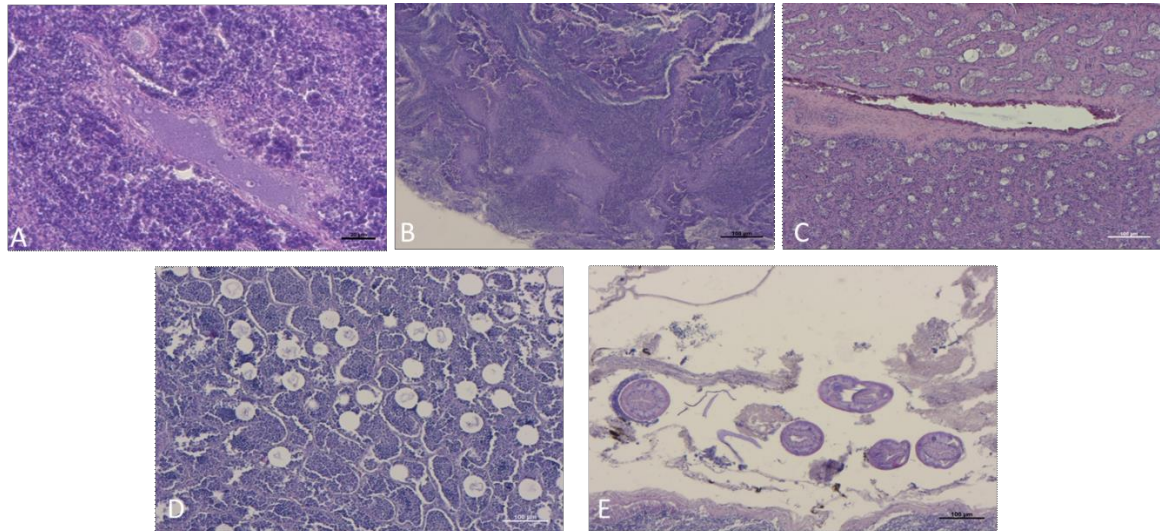
Figure 36. Histological analyzes of male gonads with alterations, such as: hemolysis (A) (scale bar= 20 μm), necrosis (B), thickening of the blood vessel wall (C), parasites in the lobules of the testes (D) and parasite outside the gonad (E). Scale bar= 100 μm.

Pearson's correlation analysis

Pearson's correlation analyses show significant positive correlations between the fish weight and the weight of gonads, and a significant negative correlation between the GSI and the reproductive stage (Table 1). Also the age of fish shows a strong positive significant correlation with fish weight and length ($p < 0.001$) and a positive but weak correlation with the reproductive stage ($p < 0.05$).

	Date	Weight (g)	Length (cm)	Gonad weight (g)	GSI	Reproductive stage	Age
Date	1,00						
Weight (g)	**-0,14***	1,00					
Length (cm)	-0,11	**0,89***	1,00				
Gonad weight (g)	**0,35***	**0,22***	0,12	1,00			
GSI	**0,39***	-0,03	-0,11	**0,96***	1,00		
Reproductive stage	**-0,30***	0,07	0,04	**-0,23***	**-0,24***	1,00	
Age	-0,01	**0,56***	**0,51***	0,06	-0,10	**0,15***	1,00

Table 1. Pearson's correlation coefficients. GSI, Gonadosomatic index. *= p ≤ 0.05; **= p ≤ 0.01; ***=p ≤ 0.001.

Fishing results

The Ancona's fleet consists of eight boats with an overall length between 24.95 and 28.6 m, a gross tonnage between 93 and 141 tons and an engine power included between 250 and 590 kW (Table 2).

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

Table 2. Characteristics of the fishing boats representing the Ancona fleet. L.O.A., length overall expressed in meters; G.T., gross tonnage; kW, kilowatt.

Considering the landing of each fishing boat, the fishing effort was assessed and the average fishing effort was determined both for the total catches (Figure 37A) and for sardines (figure 37B), and anchovies (figure 37C). The fishing effort is greater in December and March '22 for both the total catches and the two single species. Moreover, fishing effort calculated on sardines catches (Figure 37B), revealed an high peak also in August. For the fishing effort related to anchovies (Figure 37C), on the other hand, a lower fishing effort is observed for all the summer months.
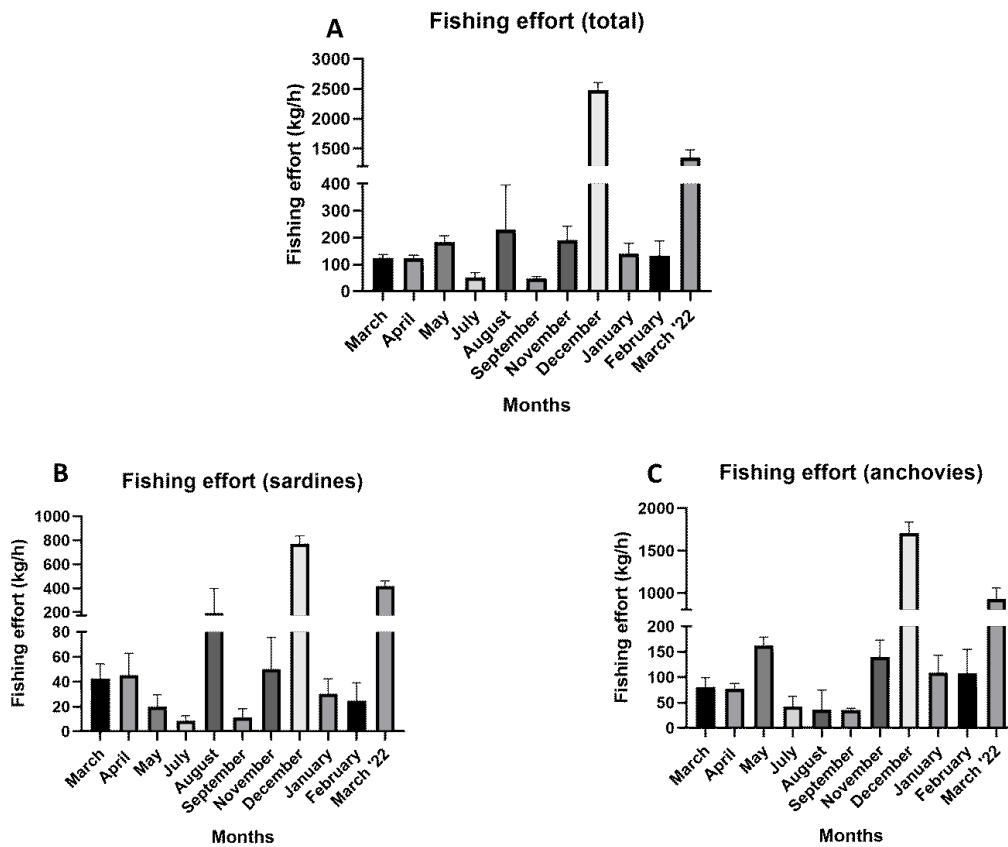
Figure 37. Fishing effort calculated on the total catches (A), on sardines catches (B) and related to anchovies catches (C).

The commercial value was also calculated monthly, both for sardines (Figure 38A) and anchovies (Figure 38B). The commercial value of sardines remains almost constant, except for December. While for anchovies there is a greater variability, ranging from a minimum of 0.89 € to a maximum of 3.85 €.
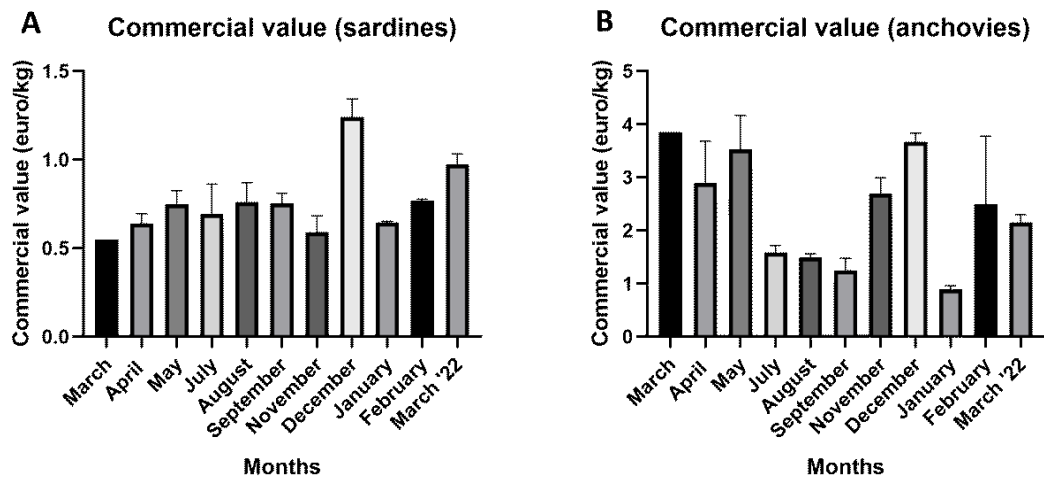
Figure 38. Commercial value of *sardines* (A) and *anchovies* (B).

Figure 39 shows the distribution of size classes for both females and males classified. As for the females, the most frequent size class is that of 13.5-13.9 cm, while for the males it is that of 13-13.4 cm.
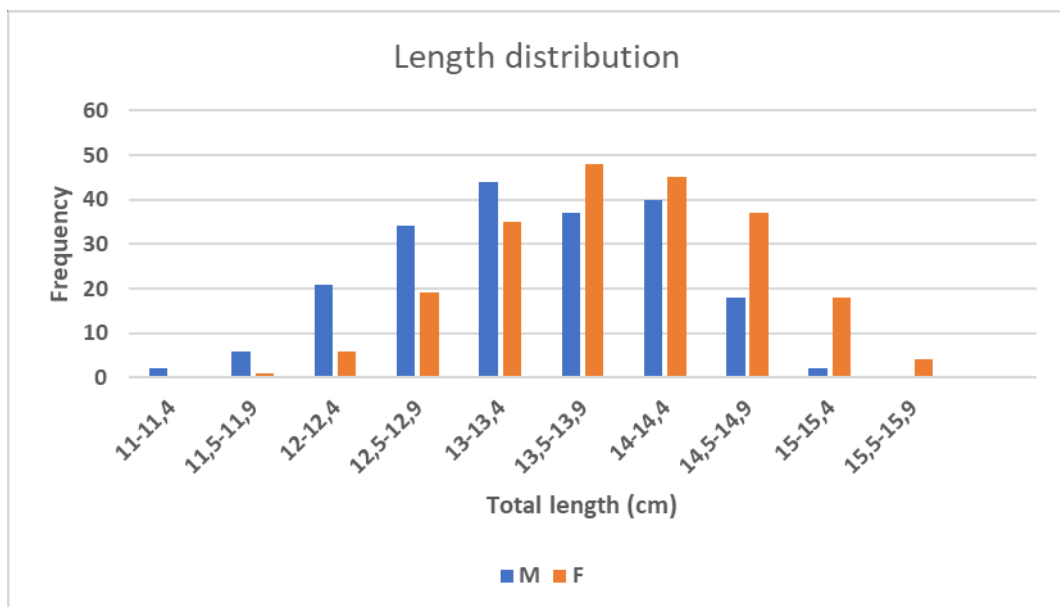
Figure 39. Length distribution of females and males classified. n = 417.

Furthermore, the distribution of sizes was observed, only of classified females, according to the fishing area. In the graphs below (Figure 40) it is possible to see how almost all the sizes are present in each fishing area. In the area 18-20 miles from Ancona (Figure 40B) the presence of the smallest size class (11.5-11.9 cm) is recorded. In the area 36 miles from Senigallia (fig. 40D), on the other hand, only larger sizes are present and in greater quantities than in the other areas.
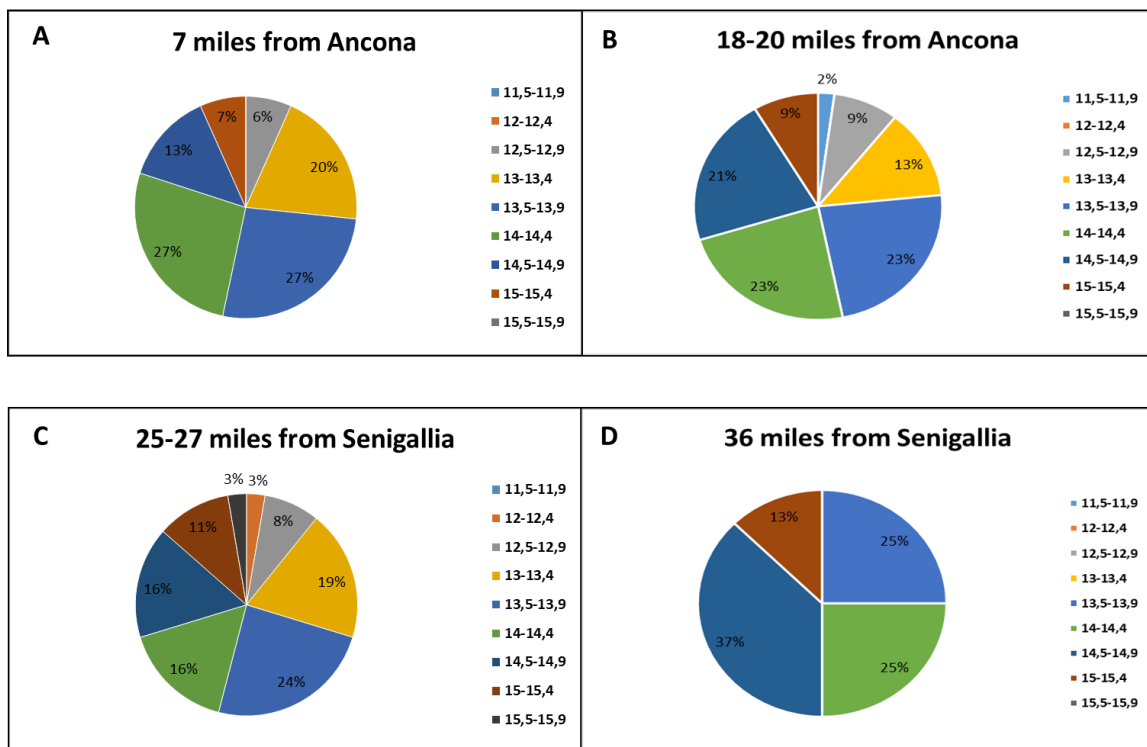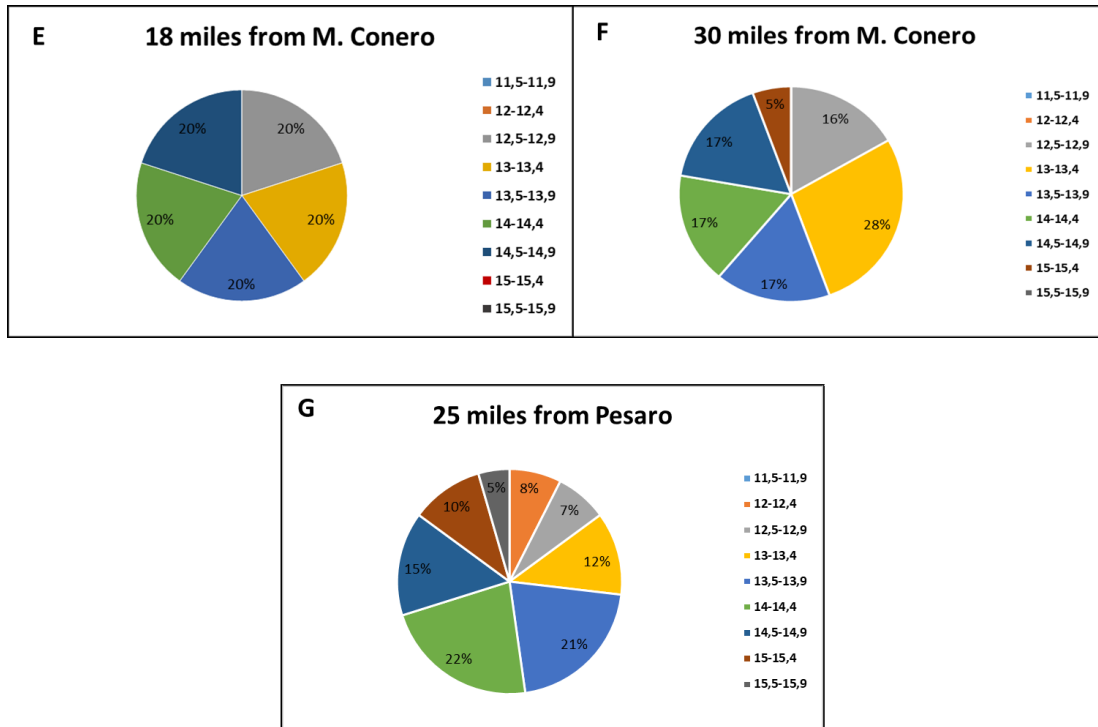
Figure 40. Distribution of ize classes of classified females according to the fishing area. 7 miles from Ancona (A), 18-20 miles from Ancona (B), 25-27 miles from Senigallia (C), 36 miles from Senigallia (D), 18 miles from Monte Conero (E), 30 miles from Monte Con ero (F), 25 miles from Pesaro (G).

Thanks to the logbooks collected, it was then observed that several species are victims of the bycatch. The month of December appears to be the one with the greatest quantity of by-catch, represented exclusively by individuals of swordfish, *Xiphias gladius* (Linnaeus, 1758). Some specimens of swordfish was then also caught in the months of April and November. In addition, two *Seriola dumerili* (Risso, 1810) and some horse mackerel, *Trachurus trachurus* (Linnaeus, 1758) were also caught in August. To these data are also added the species that were found in the cassettes in the laboratory:

- 1 mackerel, *Scomber scombrus* (Linnaeus, 1758), 3 horse mackerel, *Trachurus trachurus*, 1 *Mullus surmuletus* (Linnaeus, 1758) in March;

- 2 unidentified adult squid, 1 *Pagellus erythrinus* (Linnaeus, 1758), 1 horse mackerel, *Trachurus trachurus*, 1 *Trisopterus minutus* (Linnaeus, 1758) and 1 Serranus hepatus (Linnaeus, 1758) in May;

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

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

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

- 12 unidentified non-adult cephalopods.

Otoliths results

The length-weight relationship was determined for both males (Figure 41A) and females (Figure 41B) classified. The distribution of males starts from 11 cm up to about 14 cm, while for the females the distribution begins at 12 cm and ends at almost 16 cm. In both cases, the growth has a negative allometry, which means that they have exponential growth.
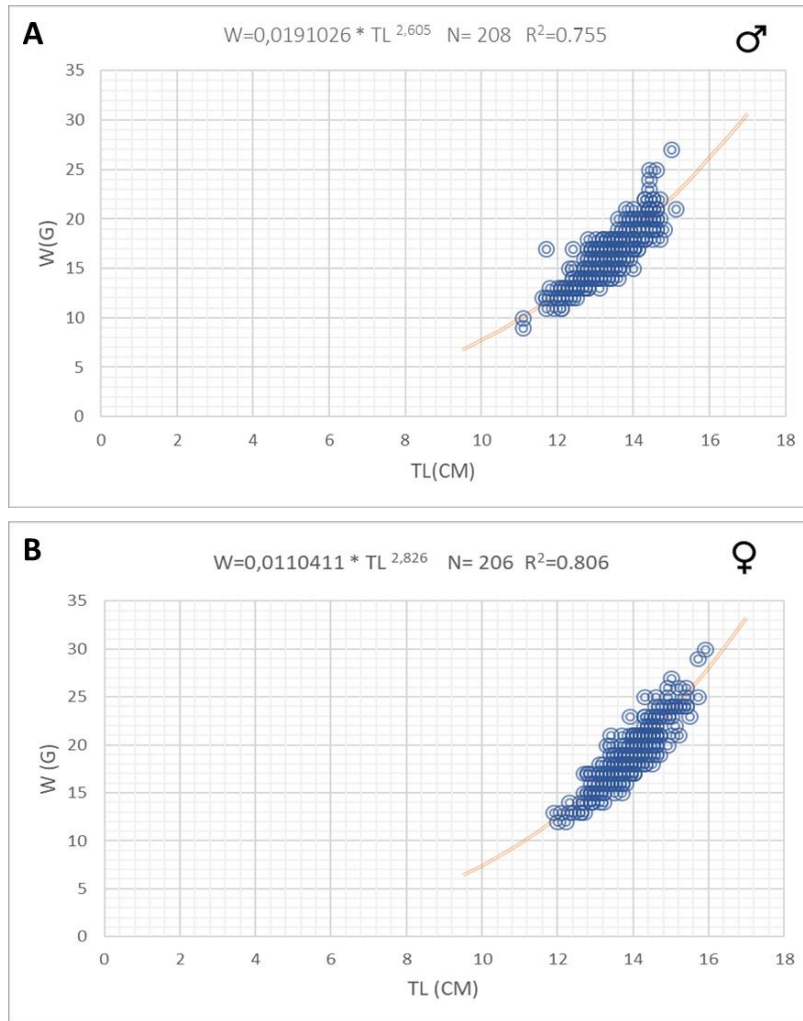
Figure 41. The length-weight relationships for males (A). The body growth of males was negatively allometric (i.e. b < 3) (t = 3,82 df = 208, p < 0.05). The length-weight relationships for females (B). The body growth of females was negatively allometric (i.e. b < 3) (t = 1,78 df = 204, p < 0.05).

The reading of the otoliths was carried out twice (Figure 42) and then the average values were calculated. The percentage of the variation coefficient (CV) and the APE are 19% and 13% respectively. The percentage of agreements is, however, 78%. The reading of the otoliths was performed twice (Figure 43) and then the mean values were calculated. The percentage of the coefficient of variation (CV) and APE are 19% and 13%, respectively.

The percentage of agreements is, however, 78%. Table 3 shows the size classes by age group (0-3). The number of specimens per year, the mean total length per year and the growth rate per year were also calculated. In this table, in fact, it is possible to see that most of the individuals (n = 396) have an age 1+ and a mean total length of 13.81 cm. On the other hand, only 1 individual is age 3 and has a mean total length of 15.00 cm. Furthermore, the growth rate is 1.00 cm for the first year, while it decreases in the following ages. It is also observed that the growth rate from age 1 to age 0 varies little due to the high variability of individuals in the population.
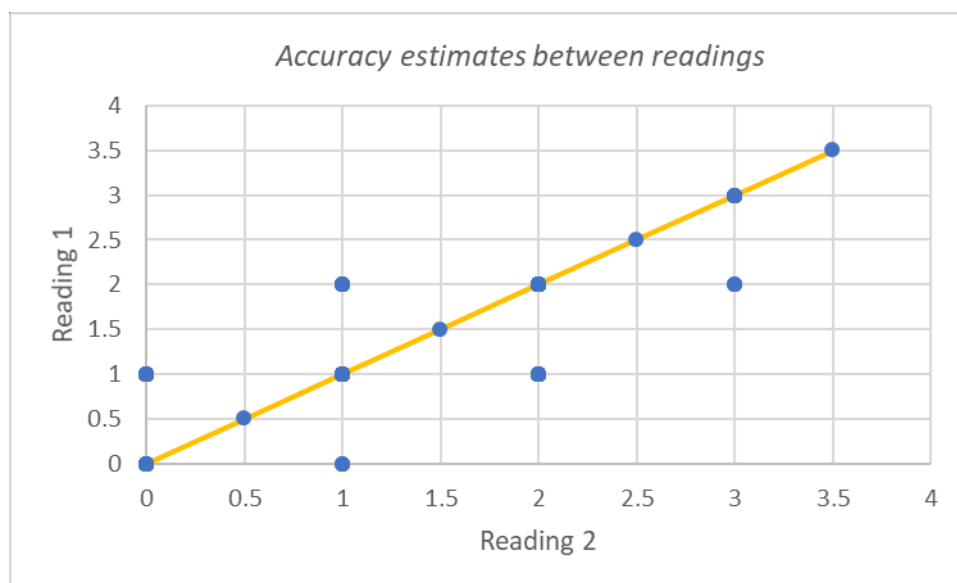
Figure 42. Accuracy between the two readings made on the otoliths. n = 396.

Age-Length keys					
TL (cm)	Age classes (Years)				
	Age 0+	**Age 1+**	**Age 2+**	**Age 3+**	*Total*
11.0	2				*2*
11.5	8				*8*
12.0	25	2			*27*
12.5	36	15			*51*
13.0	47	30			*77*
13.5	28	51			*79*
14.0		76	3		*79*
14.5		50	2		*52*
15.0		12	4	1	*17*
15.5		2	2		*4*
Total	146	238	11	1	*396*
TL mean	12.69	13.69	14.73	15.00	
Growth rate		1.00	1.03	0.27	

Table 3. Age length key for all 396 samples. The number of specimens per year, the length averages per year and the growth rate between years were calculated.

Finally, Figure 43 shows a focus of the age distribution based on the length of the classified females only. This graph shows that age 0 decreases with increasing length of individuals, while age 1 and 2 increase.
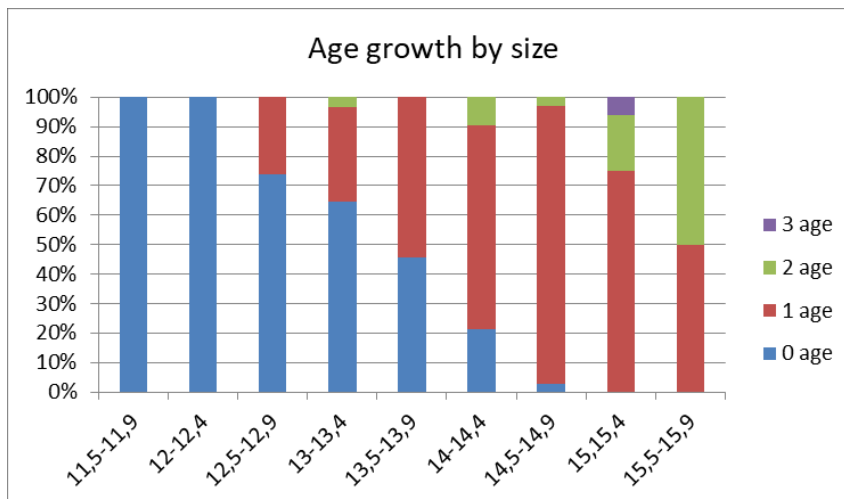
Figure 43. Age distribution based on length of classified females only. n = 207.

5. DISCUSSION

The European sardine is a key species of the marine ecosystems and plays an important role as a commercial resource in the Adriatic Sea. Therefore, studying the reproductive cycle of this species is essential to gain information on the state of wild stock and to develop a   program of fish management. Furthermore, sardines (likewise anchovies) are strongly affected by the variation of environmental conditions, and thus act as good bioindicators of climate change (Fernández-Corredor et al., 2021). However, most of the literature data on sardine reproduction in the Adriatic Sea concern the east coast, where this species has a higher commercial value compared to the Italian counterpart. For this reason, this study aims to characterize the reproductive cycle of European sardine to fill the information gap regarding the west coast.

To understand the population dynamics of a species, it is essential to determine the relationship between the sexes (Znari & Abdelaziz, 2021). Indeed, an abundance of males within a population is biologically disadvantageous since the success of recruitment depends on the presence of females (Wootton, 1982). Normally, the sex ratio depends on unequal growth and mortality rates. Furthermore, the relationship between the sexes changes

with time (seasons and years) (Znari & Abdelaziz, 2021). As for the sardine, the literature data in different areas report both a predominance of males (Mustač & Sinovčić, 2010) and females (Zorica et al., 2017). In the present work, males are predominant throughout the sampling period except in September. In this month, fishing took place in the area further off the coast (36 miles from Senigallia), where the larger size classes are present. It's interesting to note that females are mostly distributed in the larger size classes. This could be explained by the fact that larger individuals are found in the farthest waters from the coast. Indeed Sinovčić, in 2002, discovered that the sardines caught in coastal waters were smaller than those in offshore waters. The causes of this behaviour are not yet known but could be due to a different migratory behaviour between the two sexes. In addition, the numerical superiority of females in larger sizes may be the result of several factors, such as the quality or quantity of nutrients, different migratory movements, and greater vulnerability to fishing (Znari & Abdelaziz, 2021). On the other hand, a dominance of males in smaller size classes could be explained by an earlier development of testes, also due to a lower expenditure of energy to produce gametes (McBride et al., 2015).

In general, the sex ratio is mainly based on a macroscopic evaluation of gonads in the fishery resources assessment, because it allows examining a wider number of fishes and at low costs (Ferreri et al., 2009; Basilone et al., 2015). This method can be an obstacle especially when data came from only commercial fisheries (Bromley, 2003), particularly to distinguish the gonads of small pelagic fish. In this study the microscopic analysis was fundamental not only to determine the reproductive period of the sardine, but also to ascertain the determination of sex in individuals sampled during the non-reproductive months. Then histological analysises can be an excellent support to the macroscopic evaluation when the gonads are in rest as already confirmed by several authors (Smith and Walker, 2004; Sieiro et al 2014; Marisaldi et al, 2019).

The results achieved in the present study evidenced that the reproductive period goes from November to May. This is partly in agreement with what emerges from the literature. In the central-western coasts of Portugal, the reproductive period is from October to March (Nunes et al., 2011), while in the north-eastern Mediterranean area, Tsikliras and Koutrakis (2013) reported that sardines reproduce between October and March - April. Finally, in the Adriatic Sea, reproduction has been observed in the October-April period in

the last two decades (Zorica et al., 2020). This means that there has been an extension of the reproductive period from March/April to May. However, in addition to the annual variations that may occur according to the study area or genetic characteristics (Begg et al., 2005; Sinovčić et al., 2008), it must be considered that recent environmental changes, in particular the increase in temperatures, can influence the reproduction: both affecting the maturation of the gonads and the duration of reproduction (Begg et al., 2005; Sinovčić et al., 2008). The estimation of the spawning seasonality, in our results, agrees with the gonadosomatic index (GSI), which expresses gonad weight as a proportion of total or somatic weight. The GSI has been used extensively to describe the timing and duration of the spawning season (West, 1990). Despite the GSI is usually considered as a method with secondary importance in reproductive biology (Flores et al., 2019), in this study it was seen that it can also be used to estimate the maturity period as previously confirmed by Marisaldi and coworkers (Marisaldi et al, 2019). This because the gonadal weight is strongly correlated to the reproductive stages, as confirmed by Pearson correlation.

Another interesting result obtained in this study was the absence of immature females in the total samples analyzed. Probably, this is caused by the

minimum size of catch that is of 11 cm, for *Sardina pilchardus*, in according to Reg.CE 1967/2006. In fact, the length of first sexual maturity in the Adriatic Sea is about 8 cm (Sinovčić, et al., 2008). However, a recent study in the Central Mediterranean Sea reports that the size of first maturity ranges from 108 to 124 mm (total length) for females and from 102 to 122 mm for males (Basilone et al. 2021). Furthermore, in literature the age of first maturity occurs during the end of the first year of life (Sinovčić, et al., 2008). In our data, almost the total number of female specimens at the smallest size was at the developing stage and was less than a year old (age 0+, i.e. 10-11 months). This could mean that the females of this age and with this size are in their first reproductive event. Consequently, like the immature, they never reproduced. This may lead us to assume that, probably, both the age and the size of first sexual maturity are changing. Indeed, the size of first maturity in fish is strongly influences by ecological conditions, in particular the amount of available food and the temperature (Nikolsky, 1963; Blaxter, 1969; Neuheimer and Grønkjær, 2012). Moreover, the variation in size- and in age of first sexual maturity can has a direct implication on productivity fluctuations in many fish populations, conditioning for fisheries management (Morgan, 2018). From the 13-13.4 cm size class the age 1 increases and the reproductive stages are also more variable. In accordance with what was

previously said, it can be hypothesized that this size class instead represents the threshold in which individuals are larger and therefore are already facing the second reproductive season. In the size class 12.5-12.9 it emerges the presence of the regressing stage, but it is right to specify that it is a single female of 12.9 cm in length. On the other hand, from 15 cm upwards, individuals are between 1 and 3 years old and the developing and spawning capable stages return to high percentages. This could be because larger individuals, being also the oldest, have a slower reproductive capacity than younger and smaller individuals. This is also supported by some articles in the literature, which state that some fish species show a decline in reproductive capacity with aging (Patnaik et al., 1994; Žák & Reichard, 2020; Ahti et al., 2021).

During the histological analyses, several abnormalities were found in the first three stages of female reproduction. Focusing on developing and spawning capable stages, that are the most affected, what emerges from the results of this study is that there appears to be a correlation between the abnormalities and age. In fact, both for the developing and spawning capable stage, healthy females decrease with aging, while sick females increase. In the age 3 it turns out that 100% of the females are healthy, in this case, however, it must be

considered that it is only one female. The causes of these abnormalities are still unknown. However, it can be assumed that stress factors, such as pollutants or even environmental stresses, influence the correct development of oocytes. Gonadal tissue abnormalities have also been found in Atlantic herring and it is assumed that the causes may be environmental stress, active phagocytosis (Polder, 1961), physiological stress, exposure to environmental contaminants, bioaccumulation of toxic compounds, parasitic infestations (Schiedek et al., 2007; Ojaveer et al., 2015). Although it is not yet clear how the anomalies affect the future of females, it seems that these can go towards two fates: they survive, but reproductive success is compromised, or they death natural mortality (Ojaveer et al., 2015). In the latter case, this mean that natural mortality increases in the females. Other abnormalities were found in male gonads. In literature are present some studies on the infection in the testicles and liver, of *Sardina pilchardus* and *Sardinops Sagax*, due to two types of parasites: *Goussia* and *Eimeria sardinae* (Pinto, 1956; Morrison et al., 1984; Malongweni, 2016; Xavier et al., 2021). In both cases, the parasites cause coccidiosis. According to these studies, infestations with these parasites cause: necrosis, inflammatory reactions (Xavier et al., 2021) and an impaired gonad function, called "castration" by Pinto (1956).

The results of this study can be very important for fisheries management. Indeed, the determination of reproductive period, the valuation of sex ratio, the presence of abnormalities and of parasite could be potential resource to improves stock assessment and fisheries management, especially in situations of data shortage, as is the case of sardine in Western Adriatic Sea. Fishing effort was also examined in this study, calculating the amount of landed per hour. In the Adriatic Sea we talk about mixed fishing, as both sardines and anchovies are fished together. For this reason, the fishing effort was evaluated first in total (both species) and then for the two single species. Our results therefore show that, in general, the fishing effort is lower in the summer months (July, August and September). With the exception of sardines, because the month of August is still a higher fishing effort. This means that during the summer months, a lower amount of fish are caught than in the other months. Furthermore, during the summer months, anchovies also have a lower commercial value than the other months, while that of sardines remains almost unchanged throughout the year. For this reason, given the lower commercial value and the lower quantity of landed, the fishing limits could be reconsidered during the summer period for at least anchovies. In this way we could better protect the stock of anchovies, which reproduce in the summer.

6. CONCLUSION

Although the sardine is a very important species in several aspects, this is the first study on the reproduction of *Sardina pilchardus* in the western Adriatic Sea. So, because this species shows signs of collapse in the Mediterranean Sea, studies on sexual maturity, sex ratio and reproductive cycle are fundamental both in the short and long term. In the short term because they allow to better understand the biology of the sardine and, therefore, to create management plans more appropriate for the sustainability of the stock. In the long term, on the other hand, because they lead to the creation of a life-history data that is useful for comparing the different results obtained over time and observing the presence or absence of possible changes over the years. It would be interesting, then, to expand these studies not only on the commercial stock, but also on young individuals given their fragility and the importance they have on the future of the population. Furthermore, it is of great importance to deepen the knowledge on the anomalies found in both male and female gonads, so as to understand not only the causes, but also what consequences these have on reproduction and, above all, on the future of the stock.

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