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Reproduction and health status in swordfish (*Xiphias gladius*) and Atlantic Bluefin Tuna (*Thunnus thynnus*) Mediterranean stock for management of sustainable fishing

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Abstract

Swordfish (*Xiphias gladius*) and Atlantic bluefin tuna (*Thunnus thynnus*) are large epipelagic species capable of long-distance movement, as evidenced by both conventional and electronic tagging programs, and are important fisheries resources. In the Mediterranean Sea, the International Commission for the Conservation of Atlantic Tunas (ICCAT) is the reference RFMO. The ICCAT established a multiannual plan for the recovery of Mediterranean swordfish and Atlantic blue fin tuna stocks, laying down rules for conservation, management, and control to achieve biomass corresponding to maximum sustainable yield. The evaluation of health status, population structure, and reproductive dynamics underlies the conservation biology of swordfish and Atlantic bluefin tuna. In fisheries management, the application of multidisciplinary approaches (e.g., histological analysis, genomic and transcriptomic approaches, and genetic variation analysis) has proven to be an effective solution for determining the genetic structure of the population and health status of fish stocks. In this PhD project, multiple approaches were applied to better understand and shed light on the health and reproductive status of *Xiphias gladius* and *Thunnus thynnus* Mediterranean stock and support a science-based decision-making process in the context of fishery management. The detailed objectives are provided in each chapter as follows:

1. A window of vulnerability: chronic environmental stress does not impair reproduction in the swordfish *Xiphias gladius*
    - Aims: i) evaluate the cross-talk among metabolism, stress response, immune system and reproduction in immature and mature females by transcriptomic analysis; ii) identified a variation of health status related to sexual maturity and fish size by histological analysis
2. Oogenesis impairment in Swordfish (*Xiphias gladius*) caught in Central Adriatic Sea: differences between mature and immature females
    - Aims: i) evaluate the health of the liver and ovary of immature and mature females caught in the central Adriatic Sea during the summer and autumn seasons; ii) evaluate the alterations that could affect the ovaries of immature and mature females; iii) determine the range sizes landed in the central Adriatic Sea
3. ddRAD genotyping reveals new insight into the genetic structure and diversity, fitness and evolutionary potential of Atlantic and Mediterranean swordfish stocks
    - Aims: i) gain deeper insights into the genetic structure and genetic diversity at the genomic level of the three swordfish stocks, the North Atlantic, the South Atlantic and the Mediterranean; ii) assess the stock's fitness and evolutionary potential to respond to environmental changes; iii) confirm the current spatial stock boundaries and degree of mixture

4. Histological investigation on the reproductive biology of the Atlantic bluefin tuna (*Thunnus thynnus*) in the Central Adriatic Sea: differences between males and females
    - Aims: i) evaluate the gonadal status of males and females caught in the Central Adriatic Sea from February to November; ii) evaluate the alterations that could affected the ovaries and testis; iii) determine the range sizes landed in the central Adriatic Sea

INTRODUCTION

Experimental Models

Xiphias gladius

Xiphias gladius (Linnaeus 1758) is a marine bony fish and the only member of the Xiphiidae family. It is commonly known as "swordfish" for its characteristic "rostrum", which is reminiscent of a real sword. On the other hand, the scientific name Xiphias gladius comes from the Greek "ξίφος, xiphos" and from the Latin "gladius", both attributable to the term "sword". His position in the Perciform Order has been debated. The swordfish was initially associated with the Scombroid (Greenwood et al., 1966), but according to some authors, the differences would prove to be of independent origin, creating the suborder of the Xiphoidal (Gosline, 1968; Orrell & Collette, 2006). The most evident characteristic of the swordfish is the development of the upper jaw, which forms the typical "sword," which is approximately one-third of the animal's entire body. The jaw was elongated, with a length much shorter than that of the upper jaw. Morphological changes occur during ontogenesis. In the larval and juvenile stages, both jaws are equipped with small teeth, whereas in adults, they are either absent or very rudimentary. Moreover, in juveniles, the dorsal and anal fins are united with the caudal fin, while in adults, they separate into two distinct fins. The dorsal fin is triangular and sickle-shaped, with rays (about 15) arcuate and oriented backwards, while the second fin is reduced like a *pinnula*. The two anal fins show a similar shape to the dorsal fins: the first is sickle-shaped, with the tip turned back, while the second is placed in correspondence to the second dorsal fin. In adults, ventral or pelvic fins and pelvic belt are absent; instead, pectoral fins are long. The caudal fin is powerful and suitable for fast swimming; in juveniles, it is rounded and triangular, before assuming a falcated shape in adult specimens. It is connected to the body of the animal by a thin caudal peduncle with a keel on each side. *Xiphias gladius* is among the fastest fishes, reaching a maximum speed of 100 Km/h. Swordfish show a gland at the base of the rostrum, which produces a lubricating liquid that reduces its resistance to water during movement (Videler et al., 2016). *Xiphias gladius* is an obligate ram ventilation species (Wegner et al., 2010), the high speed in swimming displaces large volumes of water that cross the mouth and gills, allowing the animal to breathe. *Xiphias gladius* can reach remarkable size: it can reach 4 m in length and 500 kg in weight, as demonstrated by the 536 kg of a specimen captured in 1953 off the coast of Chile and the 550 kg of another individual landed in Cape Breton, Nova Scotia. The size of swordfish in the Mediterranean is much smaller than that in the Atlantic; in fact, specimens weighing more than 250 kg are rarely recorded. Xiphias gladius is a cosmopolitan species that is widely distributed in tropical, subtropical, and temperate waters between 45° N and 45°N, capable of long-distance movements, as documented by both pop-up satellites (Dewar et al., 2011), conventional (Neilson et al., 2009), and tags (Espíndola et al., 2011). In the Mediterranean Sea, spawning occurs during the summer, with a peak in July (Romeo et al., 2011; Marisaldi et al., 2020). The spawning areas are off Western Sicily and in the Strait of Messina (Corriero et al., 2004), the Strait of Gibraltar and the Balearic Islands (Abid et al., 2019; Macías et al., 2005; Romeo et al., 2008), the Levantine Sea (Tserpes et al., 2001; Alıçlı, 2012), and the Corsica Islands (Sardenne et al., 2022). Spawning occurs first in the Balearic Islands (Abid et al., 2019; Macías et al., 2005), later in the breeding season in the other areas of the Levantine Sea (Alıçlı, 2012), and finally in Sicily (Romeo et al., 2011). In the Atlantic Ocean, spawning occurs from April to September, and the spawning areas are in the Gulf of Mexico and along the coasts of Florida or the Caribbean Sea (Arocha, 2007; Hazin et al., 2002; Neilson, 2007).

The Atlantic and Mediterranean stocks

To manage swordfish fisheries, the International Commission for the Conservation of Atlantic Tunas (ICCAT) considers three distinct populations: North Atlantic, South Atlantic, and Mediterranean Sea. The ICCAT has established a stock boundary line that divides the North and South Atlantic stock at 5°N, and the North Atlantic and Mediterranean stock at the Strait of Gibraltar (36°N). Furthermore, the sampling areas identified by BIL-xxx were established for statistical reporting purposes (ICCAT, 2016; Neilson et al., 2013). To understand differences among the three populations, several studies were performed (Arocha, 2007; Neilson et al., 2013; Neilson, 2007; Tserpes & Tsimenides, 1995; Tserpes et al., 2009a; Righi et al., 2020; Smith et al., 2015). Using a multilocus nuclear SNP and microsatellite analysis, a shallow differentiation was found between the North and South Atlantic stocks, whereas a strong differentiation was found between Mediterranean and Atlantic stocks (Smith et al., 2015). Furthermore, according to Smith et al. (2015), the North Atlantic population extends from tropical spawning areas to 15°W (Azores Islands), whereas the South Atlantic population extends from 50°S to 20°N at 40°W and 25°N along the African coast. The Mediterranean population extends off the Strait of Gibraltar, possibly as far as 10°W. Previous studies of the Atlantic swordfish population structure have identified potential areas of mixing between the North Atlantic and Mediterranean populations off the Strait of Gibraltar (Bremer et al., 1996; Viñas et al., 2007). In the North Atlantic Ocean, the Mediterranean swordfish remains off the northwest African and Iberian coasts and occasionally migrates into the Azores (Smith et al., 2015). Atlantic and Mediterranean swordfish stocks have different growth rates (Tserpes & Tsimenides, 1995). Atlantic juveniles of swordfish grow very quickly, reaching approximately 140 cm of Lower Jaw Fork Length (LJFL) at the age of 3 years, while in the Mediterranean, juveniles reach approximately 130 cm of LJFL at the age of 5 years. For this reason, Atlantic swordfish females reach sexual maturity at a length of approximately 180 cm of LJFL, while Mediterranean females reach approximately 130 cm of LJFL. However, the most recent information by Saber et al. (2020) and Marisaldi et al. (2020) indicate an L50 (the size at which 50% of the females reach sexual maturity) of 134.3 cm LJFL in the central

Mediterranean using a histological approach. However, other authors have reported an L50 of approximately 140 cm LJFL and 170 cm LJFL in the southwestern Mediterranean Sea (de la Serna et al., 1996; Macías et al., 2005) and Strait of Gibraltar (Abid et al., 2019), respectively.

Mediterranean stock management

The Mediterranean swordfish biomass has vanished since the 1980s owing to overfishing (ICCAT, 2016). For this reason, in 2016, the ICCAT established a multi-annual plan for the recovery of Mediterranean swordfish stock, laying down rules for conservation, management, and control in order to achieve biomass corresponding to the maximum sustainable yield by 2031 (ICCAT, 2016). In particular, the ICCAT Plan introduced a total allowable catch (TAC) of 10,500 tons and a minimum catch size (100 cm), which, however, is below the first sexual maturity size of females, and a closed fishing season (January-March) to reduce the catch of juveniles. The main fishing gear used is the long liner (representing 84% of the annual catch) (ICCAT, 2016). The long liner is not a selective fishing gear for size; therefore, swordfish under the minimum catch size (100 cm or 10 kg) are caught, and only 5% can be landed and not discarded. Both the catch of juveniles and immature females (which has never been reproduced) could contribute to the collapse of the stock. A previous study conducted by Tserpes et al. (2009) evaluated the effect of four- and six-month Mediterranean-wide fishing closures. The authors suggested that the first period would result in significant (>40%) long-term increases in biomass, and the second could attain levels corresponding to the maximum sustainable yield (MSY). Furthermore, since the 1990s, a decrease in the mean catch size has been reported (Damalas et al., 2007; De Metrio et al., 1998). In the Ionian Sea, a decrease from 50 kg to 10 kg was reported, and a similar result was found in the Eastern Mediterranean Sea, with a decrease from 30 kg in 1998 to 23 kg in 2004 (Damalas et al., 2007).

Thunnus thynnus

The *Thunnus thynnus,* Atlantic bluefin tuna (ABFT), is an important fish resource. Among pelagic fishes, it represents the most important species in the world for commercialized volumes (Fromentin & Powers, 2005). The ABFT has a fusiform body, is not compressed laterally, and is characterized by the presence of two contiguous or separate dorsal fins of variable sizes. ABFT can reach remarkable dimensions, with a length that can exceed 3 m and body weight that can reach 900 kg (Fromentin & Powers, 2005). The rate of growth of juveniles is rapid, whereas it decreases in adult individuals; however, body weight increases considerably (Api et al., 2018; Fromentin & Powers, 2005). At 10 years, the ABFT generally reached dimensions of 200 cm, with a weight of 150 kg. The peculiar circulatory system of *Thunnus thynnus* allows this large pelagic to keep the temperature of the muscles, brain, and eyes warmer than the external one; in fact, it must continue to swim to produce heat and breathe (Fromentin & Powers, 2005). Bluefin tuna has a large geographical distribution: the Atlantic Ocean (from the Scandinavian Peninsula to South Africa), its adjacent seas (from the Black Sea to the Gulf of Mexico), and the Mediterranean Sea (Fromentin & Powers, 2005).

The Eastern and Western stocks

The ICCAT manages bluefin tuna fisheries considering two distinct stocks: the western stock, which generally feeds in the north-west Atlantic and breeds in the Gulf of Mexico during the spring, and the eastern stock, which uses as feeding areas the areas near Portugal, Morocco and the Bay of Biscay and the Mediterranean as spawning areas during the summer period (Muhling et al., 2013). The ABFT spawning areas are restricted to each population (around the Balearic Islands, Sicily, and Levantine Sea in the Mediterranean Sea, and the Gulf of Mexico in the Atlantic Ocean) (Corriero et al., 2020; Fromentin & Powers, 2005; Karakulak et al., 2004a; Sarà & Sarà, 2007). The reproductive biology and the health status of ABFT were studied by many authors (Corriero et al., 2005; Medina, 2020; Mylonas et al., 2010; Zohar et al., 2016). However, some aspects of their reproductive biology remain unclear. These two stocks show a different L50: in the Western stock, the L50 of females is estimated to 228.8 cm CFL (Curved fork length) (corresponding to age 8-10), while in Eastern stock, to 103.6 cm (corresponding to age 3) (Corriero et al., 2005; Corriero et al., 2020; Diaz & Turner, 2007; ICCAT, 2018; Rooker et al., 2007; Farley & Ohshimo, 2019). However, both stocks show a similar growth rates (Porch et al., 2019; Restrepo et al., 2010), fecundity and reproductive period (Knapp et al., 2014). It is likely that sexual maturity may be underestimated in both the stocks (Medina, 2020).

Eastern stock management

In the past, the high demand and absence of adequate management plans have caused excessive fishing efforts, causing considerable damage to the western stock (Porch et al., 2019). Bluefin tuna has been extensively exploited in the Mediterranean for thousands of years, but the quantities caught have become increasingly important, especially since the 1990s, because of the sharp increase in Japanese demand due to the spread of sushi-sashimi, a well-known dish based on raw fish. In 1998, purse seine vessels were twice as long and four times as powerful as they were in 1970 (Fromentin & Powers, 2005). In November 2006, ICCAT established a 15-year recovery plan for bluefin tuna stocks in the eastern Atlantic Ocean and Mediterranean Sea. A progressive reduction in Total Allowable Catch (TAC) was established from 2007 to 2014. TAC represents the maximum number of fish that can be taken from a given stock over a certain period to decrease catch. TAC varied from to 50,000-61,000 tons, between mid-90 and 2007 to 13,000 tons in 2010-2014, drastically reducing the pressure on the stock (ICCAT, 2019). The other measures used in the recovery plan concerned the reduction of the fishing season for purse-seiners, the prohibition of the use of planes or helicopters for the search of bluefin tuna, a minimum size (30 kg or 115 cm CFL), an ICCAT regional project to ensure full observer control of all vessels and transfer operations, the prohibition of transhipment of fish at sea but only in designated ports, and other controls aimed at ensuring the effectiveness of the management plan. In the Mediterranean Sea, the most widely used fishing methods for ABFT are purse-seine, long-line, tuna trap, and recreational fishing (Mylonas et al., 2010). In addition, part of the tuna caught by purse-seine are transferred live by tug to farmed cages in the open sea, which travel at a towing speed that usually does not exceed 1-1.5 knots to avoid excessive mortality and are transported to the farms. Mediterranean farms mostly use circular cages that float in the open sea. The size of the cages varied from 30 to 90 m in diameter, with the depth of the nets generally between 15 and 30 m. The fattening season, which usually lasts for several months, is closely linked to market demand. Once the tuna reaches the desired size and weight, it is sacrificed and subsequently sold, mainly to the Japanese market. During this period, bluefin tuna are mainly fed a mixed diet consisting mainly of a variety of small pelagic species (Ottolenghi, 2008).

Reproduction of teleost fish

Endocrine control of reproduction: the Hypothalamus–Pituitary–Gonad axis

In fish, the hypothalamus does not communicate directly with gonads to control reproduction. Instead, neurohormones produced in the hypothalamus affect the pituitary gland, which is the major endocrine organ in vertebrates (Fontaine et al., 2020). The hypothalamus releases gonadotropin-releasing hormone (GnRH), which in turn stimulates the gonadotroph cells of the pituitary to produce gonadotropin hormones (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which are released into the bloodstream to be transported to the gonads (Weltzien et al., 2004). This cascade of events is called the hypothalamus-pituitary-gonad axis (HPG) and is activated by external stimuli, such as temperature (Alix et al., 2020). GnRH is a 10 amino acids long neuropeptide. Three forms of GnRH have been recognized in teleosts. GnRH-1, localized in the preoptic area (POA), is a releasing hormone that stimulates gonadotropins and is essential for the onset of puberty (Zohar et al., 2010). GnRH-2 and GnRH-3 are not directly involved in regulating gonadotropin release (Kah, 2020). Several hypothalamic signals acting on GnRH neurons can inhibit and/or activate the production of GTHs, such as dopamine (DA), γ-aminobutyric acid (GABA), neuropeptide Y (NPY), and kisspeptin (Kiss) (Akazome et al., 2010; Levavi-Sivan & Yaron, 2003; Muñoz-Cueto et al., 2017). Dopamine, a catecholamine that acts as a neurotransmitter and is produced by dopaminergic neurons, inhibits GTH release, in contrast to the stimulatory effect of GnRH. Dopaminergic neurons act on GnRH neurons or interact with appropriate receptors on gonadotrophs in the pituitary gland. GABA stimulates GnRH neurons and inhibits dopamine, thus promoting the synthesis and release of FSH and LH. NPY plays a role in the control of feeding in teleosts, and may also play a role in the coordination of the reproductive and growth axes mediated by the pituitary gland. Kisspeptins are a family of peptides that operate upstream of the GnRH neurons.

FSH and LH control gonadal steroid production, the development of gonads and germ cells, ovulation, spermiation, and spawning (Levavi-Sivan et al., 2010; Zohar et al., 2010). Both hormones are heterodimers consisting of a common α subunit for both LH and FSH and a specific β subunit for LH or FSH (Pierce & Parsons, 2003). The association between the two subunits (α and β) determines the release of active hormones. LH and FSH are released into the bloodstream and transported to the gonads, where they find their receptors. However, the effect of GTHs at the level of the gonads depends on the density of the receptors for FSH and LH in the target cells, the steroidogenic cells (Leydig cells) in the testis, and the theca and granulosa cells of the ovary. Steroidogenic cells synthesize sexual hormones such as 17β-estradiol (E2) and 11-ketotestosterone (11-KT), the two main female and male sex hormones in fish, respectively, and 17α,20β progesterone (Nagahama, 2002). With the synthesis of steroid hormones, HPG is fully activated and gametogenesis proceeds.

Oogenesis

Oogenesis is the developmental process by which oogonia are transformed into mature oocytes, 100-200 times larger than the starting cell and ready to be spawned (Wootton & Smith, 2002; Lubzens et al. 2010) (Figure 1). During oocyte growth, many changes occur in terms of the biochemical composition, size, transcription, and water content (Lubzens et al., 2017). For this reason, it is suitable to divide oogenesis into the primary and secondary growth phases, with vitellogenesis as a marker of transition between these two phases.

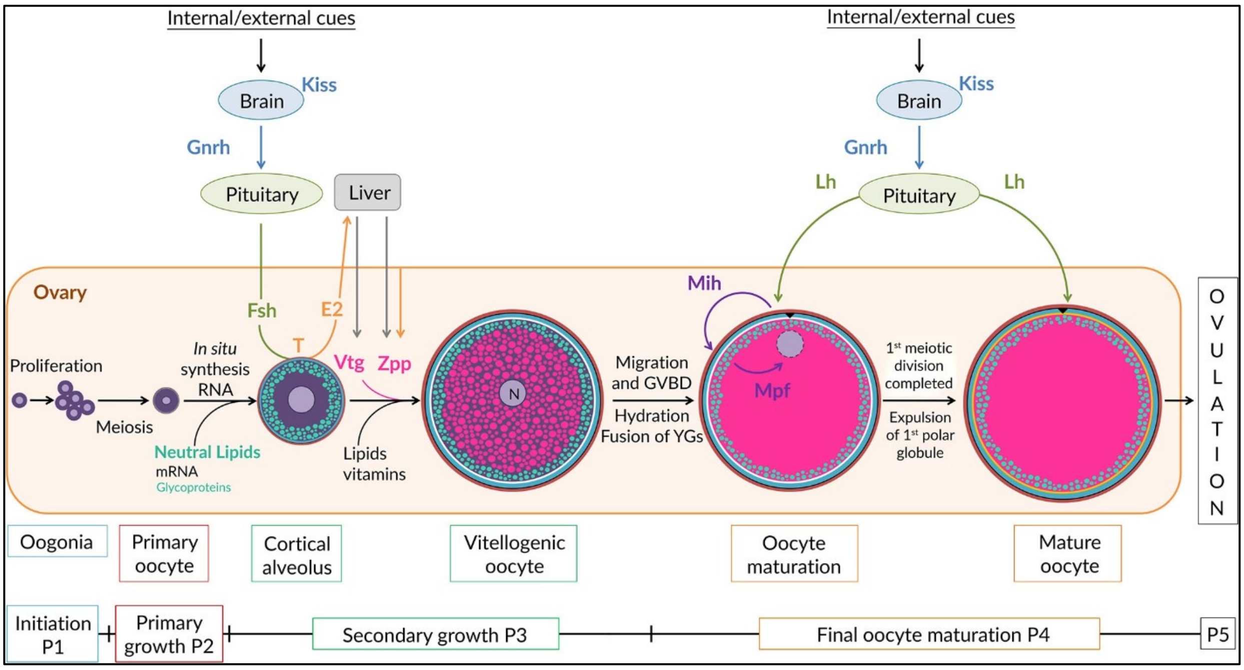

Figure 1 - Schematic representation of oogenesis. Reproduced from Alix et al., (2020)

Proliferation of oogonia and primary growth phase

The first stage of oogenesis involves the transformation of PGCs into oogonia (Nagahama and Yamashita 2008; Lubzens et al. 2010). Oogonia proliferate through mitotic division and form oogonial nests. Within a nest, oogonia are connected by cytoplasmic links, and each nest is enclosed by a thin layer of somatic cells, the precursors of granulosa cells. Endocrine control at the earliest stages of oogenesis is not well-defined. This proliferation can be stimulated by 17β-estradiol (E2) (Miura et al., 2007), but the origin of E2 at this stage of oogenesis is not yet clear. In *Ciprinus carpio* and *Hucho perryi*, the authors suggest that mitotic proliferation is activated by progesterone 17α,20β-P, E2, while meiosis is initiated by Maturing Inducing Hormone (MIH) (Higashino et al., 2003; Miura et al., 2007). Following mitotic proliferation, some oogonia gradually enter meiosis and differentiate into primary oocytes, while the remaining part continues to divide by mitosis, forming a reserve of oogonia for the next spawning season in iteroparous species. After meiosis begins, the primary oocyte enters prophase and subsequently arrests in diplotene I. At this stage, the chromosomes appear "lampbrush”, not yet condensed. Inside the nucleus of the primary oocyte, the nucleoli became strongly visible. In the cytoplasm, there are darker structures called Balbiani’s vitelline bodies, which characterize an area of strongly basophilic cytoplasm where ribosomes and proteins accumulate (Menn et al., 2007). Granulosa and theca cells are formed, which are separated by a distinct basement membrane, and the formation of the follicle complex is completed (Wootton & Smith, 2002; Lubzens et al., 2010). Granulosa cells and oocytes begin to separate, and in the space formed, the oocyte extends microvilli, which allows a high exchange of hormones and macromolecules. Around the microvilli, a protein structure called the zona radiata begins to lay down (Menn et al., 2007). The microvilli maintain contact with granulosa cells outside the zona radiata. The formation of cortical alveoli, membrane-limited vesicles of variable size that originate from the Golgi apparatus, is rich in proteins, carbohydrates, and calcium deposits (Golpour et al., 2016; Shibata et al., 2012) (Figure 2).

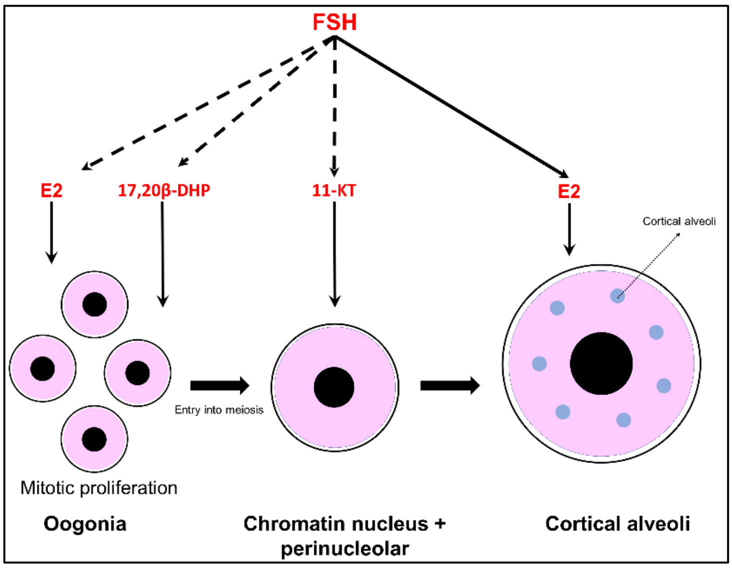

Figure 2 - Schematic representation of the proliferation and primary growth phases.

The contents of cortical alveoli are essential after fertilization to block the entry of other spermatozoa and prevent polyspermy. The formation of cortical alveoli is stimulated by E2 and the HPG axis is completely activated, marking the onset of puberty (Okuzawa, 2002). After the formation of the cortical alveoli, in some species of teleosts, that is tuna, lipid droplets become evident inside the oocyte; neutral lipids are used as energy reserves (Medina et al., 2002). It is likely that the lipid droplets consist of very low-density lipoproteins (VLDL), which are rich in triglycerides, and low-density lipoproteins (LDL), which are synthesized by the liver, released into the bloodstream, and incorporated by the oocyte (Endo et al., 2011). There are two hypotheses on how neutral lipids are incorporated in oocytes:1) VLDL is processed by ovarian lipoprotein lipase (LPL) into low-density lipoproteins (LDL), and the free fatty acids (FFAs) are released from triacylglycerol (TAG) that are engulfed by the oocytes and resynthesized as lipid droplets; 2) VLDL binds the lipoprotein receptors and is endocytosed into the oocyte before the removal of FFAs, which are then used for lipid droplet formation (Hiramatsu et al., 2015; Lubzens et al., 2010). In some species of teleosts with pelagic eggs, including *Xiphias gladius*, lipid droplets are important for the buoyancy of the eggs, favoring oocyte fertilization. The size, color, distribution, and amount of lipid droplets are species-specific, making them a biomarker that can discriminate and identify the species (Wootton & Smith, 2002).

Vitellogenesis and secondary growth phase

The secondary growth phase begins after the growth of the primary oocytes. The key characteristic of this stage of oogenesis is vitellogenesis (Ding 2005; Pankhurst 2008; Lubzens et al. 2010). In fact, granulosa cells, under the stimulus of FSH, produce estrogen, particularly 17β-estradiol (or E2). Estrogen, released in the bloodstream, reaches the liver, binds to its nuclear receptor inside hepatocytes, and stimulates the transcription of vitellogenin genes (Carnevali et al., 2006; Hiramatsu et al., 2015). E2 has three main effects on the liver:1) stimulates the synthesis of ERs, 2) stimulates the expression of *vtg* genes, and 3) stimulates the expression of zona radiata protein (ZPs) genes. Vitellogenin (VTG) is a phospholipoglycoprotein with high molecular weight (300-640 Kda). VTG is a precursor of yolk proteins and is present only in females. VTG is characterized by five domains:1) lipovitellin heavy chain (LvH), 2) phosvitin (Pv), 3) lipovitellin light chain (LvL), 4) β'-component (β'-c), and 5) C-terminal peptide (Ct). The first is the largest domain that provides the embryo with phospholipids and amino acids (Reading et al., 2009). VTG is transferred from the liver to the ovaries in the bloodstream and arrives at thecal blood vessels. From theca blood vessels, vitellogenin reaches the oocyte surface through the pore canals of the zona radiata and is endocytosed by receptor-mediated endocytosis involving clathrin-coated pits of vesicles (Hara et al., 2016). Once vesicles containing vitellogenin move into the ooplasm, they fuse with lysosomes to form multivesicular bodies (Wallace & Selman, 1990), where vitellogenin is cleaved into smaller yolk proteins by lysosomal enzymes (cathepsins). After cleavage, the final yolk proteins were phosphovitin, lipovitellin, β-components, and terminal peptide C. Lipovitellin consists in two polypeptides LVA and LVB*,* represents an important source of lipids and amino acids that are indispensable during embryonic development. Vitellogenin is not the only molecule that females transfer into oocytes; there are also neutral and polar lipids, such as phosphoglycerols, triacylglycerols, vitamins, and minerals (Fuiman & Faulk, 2013). Moreover, between the network of microvilli that connects the oocytes to the cells of the granulosa, there are gap joints that form aqueous channels, allowing the passage of ions and other small molecules. During the secondary growth phase, the mass of the gonads accounted for up to 20% of the total weight. At the same time, the zona radiata form a real shell able to protect the oocyte avoiding that, once ovulated, it is injured (Figure 3).

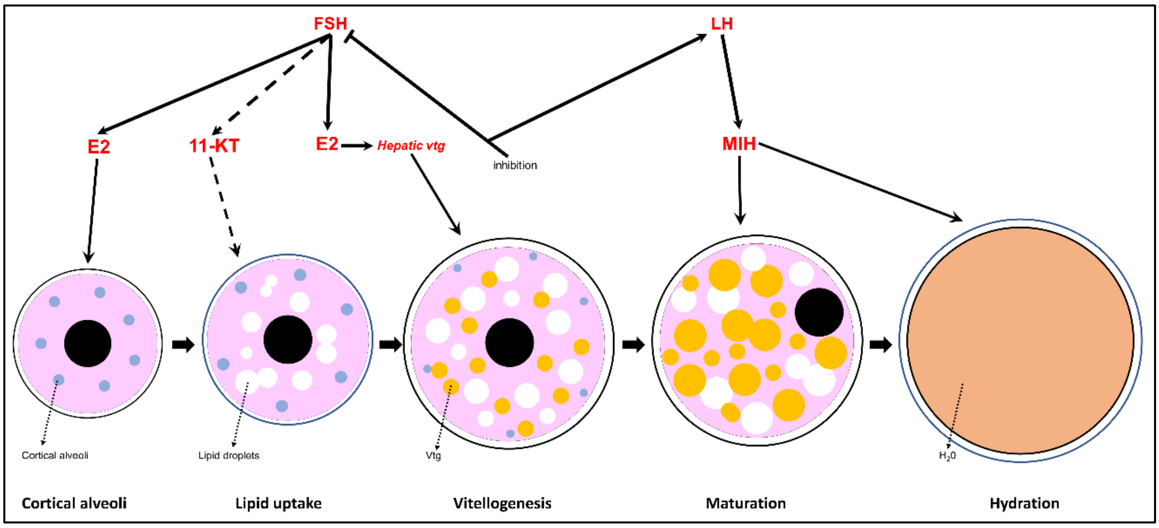

Figure 3 - Schematic representation of the secondary growth phase of oogenesis and final oocyte maturation

Oocyte maturation and ovulation

After vitellogenesis, the maturation phase begins, marked by four main events:1) a steroidogenic shift from E2 to the maturation-inducing hormone (MIH); 2) the resumption of meiosis that was blocked in diplotene I and extrusion of the first polar body; 3) coalescence of lipid and vitellogenin globules; and 4) water from the outside to hydrate the oocytes. Meiosis stops again in metaphase II and is completed only after fertilization by a sperm (Kagawa, 2013; Berta Levavi-Sivan & Yaron, 2003; Yoshitaka Nagahama & Yamashita, 2008). Maturation is initiated by LH secretion from the pituitary gland, while FSH levels decrease. Teca cells produce 17α,20β dihydroprogesterone, the MIH. However, in response to the presence of MIH, oocytes must present membrane progestin receptors (mPRs) in the oolemma, which corresponds to the acquisition of follicle maturational competence (Thomas et al., 2006). After the binding of MIH to mPRs, a signalling cascade promotes meiotic resumption by activating maturation-promoting factor (MPF), the final inducer of meiosis resumption and oocyte maturation (Clelland & Peng, 2009; Lessman, 2009; Pang & Thomas, 2010; Urbatzka et al., 2011). Meiosis is resumed, and the nucleus of the oocyte (germinal vesicle) migrates towards the animal pole, taking up a position in the periphery of the ooplasm. After reaching the animal pole, the germinal vesicle breaks down with the dissolution of the nuclear membrane. This dissolution process is required for meiosis. Meiosis I is now completed, with the formation of two, unequally sized cells, the large oocyte and a small first polar body, which degenerate. The oocytes then enter meiosis II until they are arrested at metaphase II. The final stage of maturation is hydration, which is the main characteristic of teleosts (Figure 3). Oocyte hydration depends on molecular water channels, aquaporins, and the difference in osmotic pressure after the cleavage of vitellogenin in single amino acids. Aquaporins are proteins that transport water along an osmotic gradient (Fabra et al., 2005). Mature oocytes are released into the ovarian lumen during ovulation.

The follicle wall breaks down and opens the follicle into the ovarian lumen. Follicle wall rupture is caused by a protease system that digests the extracellular matrix (ECM) in the follicle (Takahashi et al., 2019). At ovulation, only the oocyte is released into the ovarian lumen, and granulosa and theca cells remain within the ovary. Follicular cells become a continuum with epithelial cells, losing their shape as a result of their collapse after oocyte release. After ovulation, the follicle is called the post-ovulatory follicle (POFs). POFs gradually collapse, while granulosa cells decrease in size and theca thickness (Corriero et al., 2021b; Quirk et al., 2004; Yalnız & Yilmaz, 2019).

Atresia

In vertebrates, many ovarian follicles that are recruited for development do not completely mature, degenerate, and are reabsorbed in a process known as follicular atresia. Atresia is a physiological process that occurs within the ovary (Babin et al., 2014; Corriero et al., 2021) and facilitates the recycling of unused cellular components (Wootton & Smith, 2002; González-Kother et al., 2020). Atresia can occur at all stages of oogenesis but mainly in the vitellogenic phase. Atresia consists of four stages: alpha (α), beta (β), gamma (γ), and delta (δ). In vitellogenic oocytes, the alpha stage consists of lysis of the nuclear envelope with the dispersion of nuclear content in the ooplasm. The zona radiata begins to fragment and break down, while a small number of follicular cells degenerate by apoptosis. Granulosa cells undergo hypertrophy after the breakdown of zona radiata and begin phagocytosis of the yolk. The lipid content starts to coalesce under the action of hydrolytic enzymes and the yolk granules liquefy. During this phase, the oocyte showed an irregular shape. In the beta stage, follicular cells appear disorganized. In oocytes with abundant lipid droplets, residual vacuoles were distributed throughout the atretic follicles. At the end of beta atresia, the atretic follicles may progress through the following stage of atresia or can be completely reabsorbed or passed directly at the final stage of atresia. Apoptosis of follicular cells occurs in late atretic follicles. In the gamma stage, granulosa cells are characterized by the presence of light-yellow material in the cytoplasm and irregularly shaped nuclei. Phagocytosis of oocyte components is still active in follicular cells, and the number of theca cells and blood vessels is strongly reduced. In the delta stage, granulosa cells were drastically reduced in number and were not surrounded by thecal cells and blood vessels. At the end of this phase, atretic follicles and other residuals are reabsorbed (Corriero et al., 2021b; Quirk et al., 2004; Yalnız & Yilmaz, 2019). The presence of unyolked atretic follicles has been reported in many fish species (Corriero et al., 2003; Corriero et al., 2021b). Atresia affects previtellogenic oocytes at the perinucleolar and lipid-cortical alveoli stages with similar morphology (Corriero et al., 2003; Corriero et al., 2021b; Miranda et al., 1999).

Spermatogenesis

Spermatogenesis is a process during which a small number of diploid spermatogonia cells develop into many highly differentiated haploid spermatozoa (Figure 4).

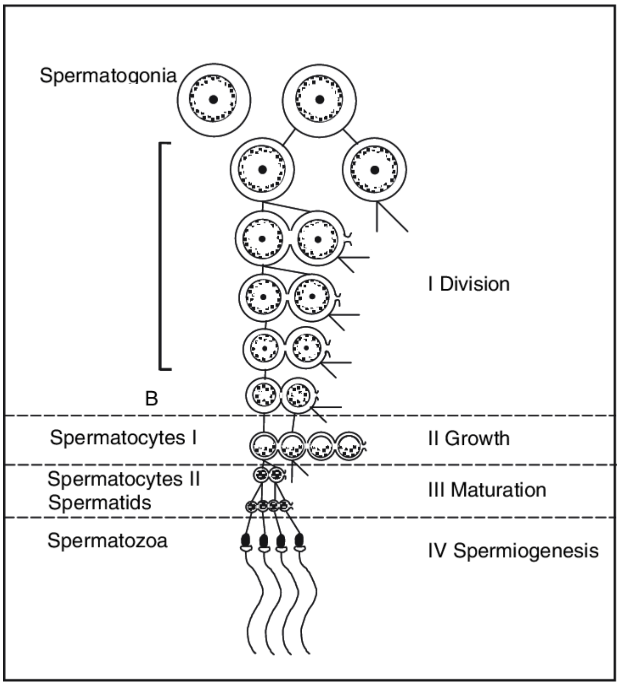

Figure 4 - Schematic representation of spermatogenesis. Reproduced from Makeyeva 1992

Regarding oogenesis, the temporal sequence of spermatogenesis is regulated by gonadotropins secreted by the pituitary gland, FSH, and LH, the effect of FSH tend to dominate the earlier stages of spermatogenesis, and LH plays a role later in the process. Sertoli and Leydig cells are the two major somatic cells characteristic of testicular architecture and function. Leydig cells are regulated by both FSH and LH, whereas Sertoli cells are primarily regulated by FSH. Leydig cells produce and secrete 11-KT in response to LH, whereas Sertoli cells are essential for spermatogenic spermatocysts development. The basic functional unit of the spermatogenic epithelium in fish is a spermatocyst formed by a dynamic group of Sertoli cells surrounding the germ cells, which are in different stages of spermatogenesis. Two types of spatial arrangement are described. In the first type (restricted spermatogonial distribution), the distal regions of the germinal compartment, near the tunica albuginea, are occupied by Sertoli cells surrounding early, undifferentiated spermatogonia. As the cells divide and enter in meiosis, the cysts migrate towards the region of the spermatic ducts located centrally in the testis, where spermiation occurs. In the second type (unrestricted spermatogonial distribution), spermatogonia spread along the germinal compartment of the testis. Cysts do not migrate or are displaced during development (Schulz et al., 2010) (Figure 5).

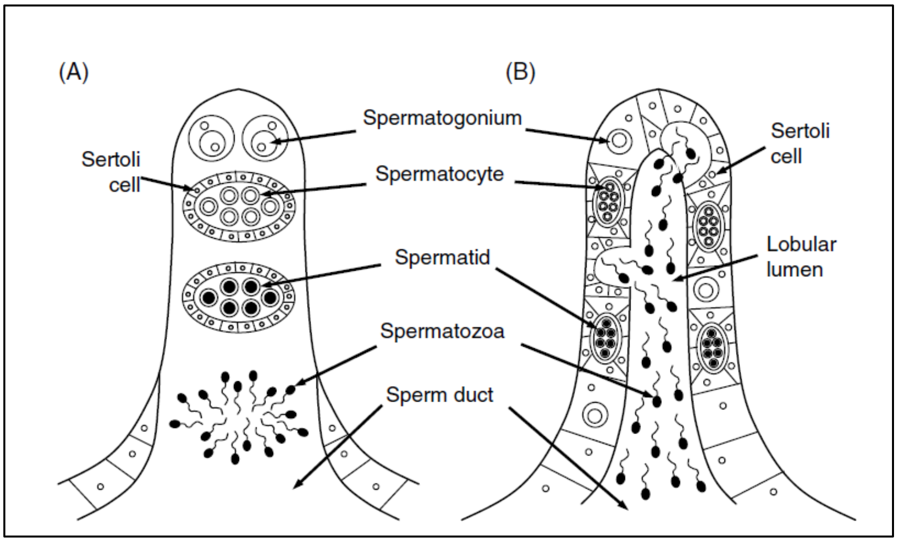

Figure 5 - Spatial arrangement of testes: (A) restricted spermatogonial distribution; (B) unrestricted spermatogonial distribution (Reproduced from Wootton & Smith, 2002).

Each spermatocyst has two cellular components: Sertoli cells, which are derived from somatic cell precursors in the germinal epithelium and are comparable to the granulosa cells of the ovarian follicle complex and germ cells derived from PGCs. The number of Sertoli cells is species-specific (Schulz et al., 2010). Tight gap junctions ensure that germ cells are completely isolated from cells outside the spermatocyst. Spermatogenesis can be divided into three different phases: the mitotic or spermatogonial phase with different generations of spermatogonia (i.e., undifferentiated spermatogonia, including stem cells, and differentiated or differentiating spermatogonia), the meiotic phase with primary and secondary spermatocytes, and the spermiogenic phase with haploid spermatids emerging from meiosis and differentiating, without further proliferation, into motile and flagellated spermatozoa.

The first phase of spermatogenesis is defined by the proliferation and differentiation of diploid spermatogonia via mitotic division (Schulz et al., 2010). This proliferation is dependent on androgens and is typically stimulated by 11-KT stimulation. Androgens essentially act as paracrine factors that influence Sertoli cells, which have androgen receptors (ARs) lacking in spermatogonia. The action of androgens is mediated by growth factors produced by Sertoli cells, including IGF-I and activin, which stimulate mitosis in spermatogonia. Levels of plasma FSH show a peak during proliferation, with FSH stimulating androgen synthesis by Leydig cells. During this phase, undifferentiated spermatogonia (type A_{und}) produce differentiated type A spermatogonia (A_{diff}). After this phase, A_{diff} undergoes an irreversible commitment to differentiate into B-type spermatogonia. In the transition from A_{und} to B-type spermatogonia, morphological changes occur in the cells (Schulz et al., 2010). Type B spermatogonia are smaller and have a smaller nucleus, and differentiation can be made based on cell/nuclear size and the number of cells per cyst into early and late type B spermatogonia (B_{early} and B_{late}) (Figure 6).

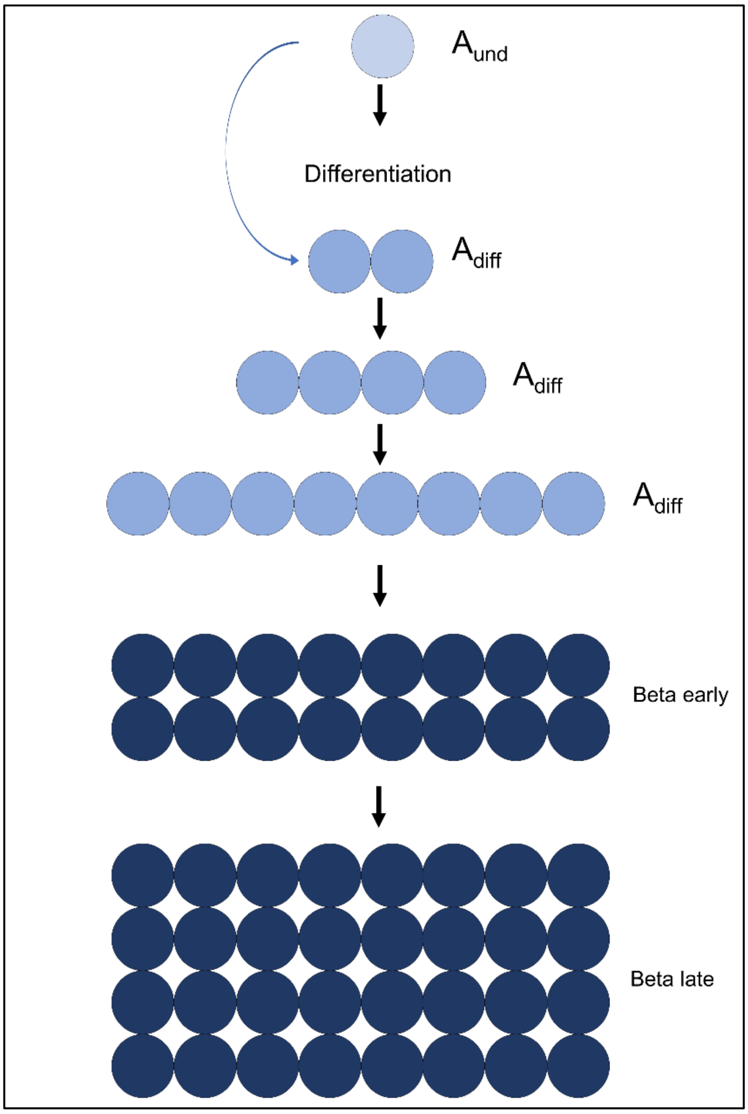

Figure 6 - scheme of the proliferation and differentiation of the diploid spermatogonia by mitotic divisions

Type B spermatogonia divide more rapidly than type A spermatogonia during mitosis. At the end of the mitotic division cycle, each spermatogonia type B differentiates into primary spermatocytes that enter meiosis I. The transition from mitotic to meiotic division of germ cells in a spermatogenic cyst is triggered by a brief surge in LH, which leads to the production of 17α,20β-P. This steroid is essential for the initiation of meiosis and, hence, the formation of primary spermatocytes. However, once meiosis has started, further development of spermatocytes is dependent on the presence of androgens, usually 11-KT, once again controlled by FSH acting on the Leydig cells. The features of meiosis I include duplication of DNA and recombination of genetic information. Two secondary spermatocytes resulting from meiosis I were produced, each with a haploid number of chromosomes. These secondary spermatocytes rapidly passed through meiosis II, which generated four haploid spermatids (Figure 7).

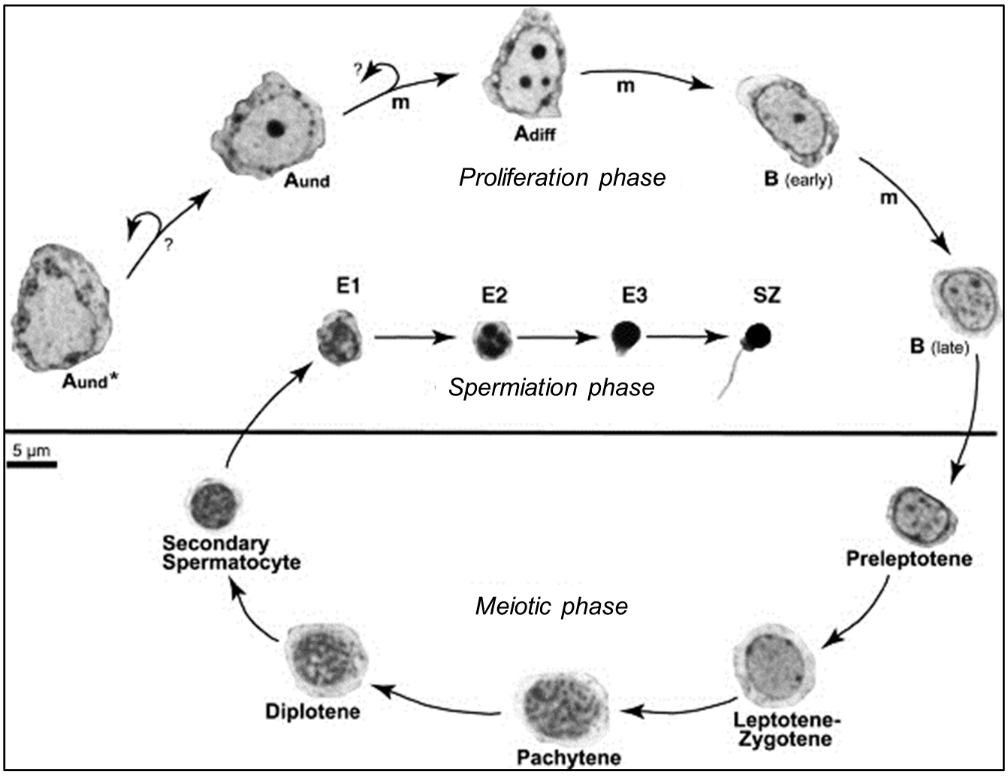

Figure 7 - scheme of spermatogenesis: proliferative, meiotic and spermiation phase (Reproduced from Schulz et al., 2010)

It is unclear whether the effect of androgens is continuous or whether their presence at certain critical times during spermatogenesis is sufficient. Haploid spermatids undergo spermiogenesis, a major morphological rearrangement that leads to spermatozoa production. Spermiogenesis is divided into four stages.

1. The early spermatids are linked by cytoplasmic bridges. Each spermatid has a spherical nucleus, but the nuclear membrane has a shallow groove or fossa on one side of the nucleus, where the centrioles are eventually located.
2. The chromatin in the nucleus of the spermatid is more compact and the spermatid is smaller than that in early spermatids. Two centrioles developed and were located in the fossa. The flagellum begins to form, originating from the distal centriole.
3. The chromatin in the nucleus was even more compact. The flagellum is linked to the distal centriole and is surrounded by mitochondria.
4. The nucleus was a regular sphere with condensed chromatin. The flagellum is attached approximately 110° to the head of the spermatozoa. Excess cytoplasm, called the residual body, is ejected and phagocytosed by Sertoli cells.

During the third and fourth stages, spermatids lose their intercellular links and association with Sertoli cells.

At the completion of spermiogenesis, the links between the Sertoli cells are weakened. Once a spermatogenic cyst is full of spermatozoa, the next phase is spermiation with rupture of the spermatocyst wall. Plasma levels of LH increase, resulting in the synthesis of 17α,20β-P, while 11-KT levels continue to be high. Under the influence of these steroids, the spermatocyst wall breaks down, releasing the spermatozoa into the testis tubules. The final stage in the development of a spermatozoon capable of fertilizing an egg is capacitation, including the acquisition of motility (Miura et al., 1991). These changes are physiological and do not require morphological changes (Schulz et al. 2010). This final maturation of spermatozoa can take place in the testicular ducts, gonoducts, and the external environment. The final acquisition of motility can be a response to a change in the osmotic environment, pH, or concentration of inorganic ions, such as K+ or Ca2+ (Kinsey et al., 2007). Chemical cues from eggs may also play a role in promoting sperm motility.

Reproduction of *Xiphias gladius*

Xiphias gladius is a gonochoristic species with external fecundation, with specialized germinal cells, spermatozoa, produced by testis, and eggs, produced by ovary. No external characteristic allows males to be distinguished from females, even if the latter usually reaches greater dimensions. In *Xiphias gladius*, the gonads are paired organs located lateral to the intestine, and the visual assessment of ovaries and testes follows the ICCAT guidelines shown in Table 1 (ICCAT, 2003).

Stage	**Criteria**	
	Males	**Females**
1	Gonads small ribbon-like, not possible to determine sex by gross examination	Gonads small ribbon-like, not possible to determine sex by gross examination
1	**Immature**: testes extremely thin, flattened and ribbon-like, but sex determinable by gross examination	**Immature**: gonads elongated, slender, but sex determinable by gross examination
2	Enlarged testes, triangular in cross section, no milt in central canal	**Early maturing**: gonads enlarged but individual ova not visible to the naked eye
3	**Maturing**; milt flows freely if testes pinched or pressed	**Late maturing**: gonads enlarged; individual ova visible to the naked eye
4	**Ripe**; testes large, milt flows freely from testes	**Ripe**; ovary greatly enlarged, ova translucent, easily dislodged from follicles or loose in lumen of ovary
5	**Spent**; testes flabby, bloodshot, surface dull red, little or no milt in central canal	**Spawned**; includes recently spawned and postspawning fish, mature ova remnants in various stages of resorption, and mature ova remnants about 1.0mm in diameter

Table 1 - Maturity stages for the macro examination of gonads.

The female *Xiphias gladius* has a grouped-asynchronous ovary, exhibiting oocytes at all stages of maturation: oogonia, primary growth oocytes, vitellogenic oocytes (primary, secondary, and tertiary), migratory nucleus oocytes, and hydrated oocytes (Saber et al., 2020). According to Saber et al., (2020), the ovarian classification is based on the key histological features: 1) Immature, 2)

Developing, 3) Spawning Capable, 4) Spawning, 5) Postspawning or Regressing, 6) Regenerating. The classifications based on the histological features are summarized in Table 2.

Maturity status	**Phases**	**Microscopic features**
Immature	**Immature**	Only oogonia, primary growth or cortical alveoli oocytes; absence of POFs; no atresia; thin ovarian wall and little space between oocytes
Mature	**Developing**	Primary vitellogenic oocytes; absence of POFs; some alpha and/or beta atresia may be present; little space between oocytes
Mature	**Spawning Capable**	Secondary and tertiary vitellogenic oocytes; absence of POFs; atresia (<50%) can be present
Mature	**Spawning**	Migratory nucleus or Hydrated oocytes; absence or presence of POFs; atresia (<50%) can be present
Mature	**Postspawning Regressing**	primary growth, cortical alveoli and primary vitellogenic oocytes; absence of POFs; abundant alpha and/or beta atresia (<50%); disorganization of ovary structures, with spaces; thick and/or wrinkled gonad wall is observed
	Regenerating	primary growth oocytes; absence of POFs; Late stages of atresia; some spaces between oocytes; thick and/or wrinkled gonad wall is observed
*The presence of POFs reflects spawning ovaries		

Table 2 - Microscopic maturity classification for swordfish females according to Saber et al. (2020). POFs, postovulatory follicles.

The ovary can contain 1 to 29 million eggs. Arocha (2007) estimated that Atlantic swordfish release oocytes every three days, for a total of about 81 spawning events per spawning season. This process is also known as *batch deposition*. The number of eggs released per reproductive event depended on the size of the organism. Although the swordfish is an almost solitary species, during the breeding season it tends to aggregate in couple (Mejuto and García-Cortés 2014; Romeo et al. 2009) and to migrate towards "spawning areas" in warm waters. In the Atlantic, spawning occurs in spring in the equatorial-tropical zone (Caribbean and Gulf of Mexico), whereas in the Mediterranean Sea, the reproductive season extends from June to September. In the Mediterranean Sea, spawning occurs during the summer season with a peak in July (Megalofonou et al., 1995; Romeo et al., 2008), and the main spawning areas are in Western Sicily and in the Strait of Messina (Corriero et al. 2004), in the Strait of Gibraltar and the Balearic Islands (Abid et al., 2019; Corriero et al., 2005; Macías et al., 2005; Romeo et al., 2008), in the Levantine Sea (Alıçlı, 2012), and Corsica Island (Sardenne et al., 2022). Swordfish reproduce cyclically every year, synchronizing the release of the gametes with the moon phases, so that the hatching of the offspring takes place during the full moon. The higher intensity offshore currents during the full moon supports larval dispersion, but the presence of phytoplankton blooms also guarantees an adequate amount of food for the larvae (Rooker et al., 2012). Embryonic development is rapid, after two-three days after fecundation, and hatching takes place. The larvae were 4 mm in size, with morphological, physiological, and ecological characteristics different from those of adults. The larvae present only the first dorsal fin and the rostrum is absent, which will appear in the following weeks. During the first stages of life, larvae live close to the water surface and feed on plankton. During development, the body grows and the rostrum begins to form. The juveniles of *Xiphias gladius* are different from those of adults: the jaw grows to form a small sword, while the mandible appears as a line of teeth that will then be completely absent in adults. The juveniles showed small spiny scales on each side of the dorsal and anal fins that disappeared in the adults. Furthermore, larvae have elongated bodies that are strongly pigmented, whereas juveniles show a blue dorsal livery evidenced by seven light blue vertical bands of uneven width, extending from the head to the tail fin (Palko et al., 1981).

Reproduction of *Thunnus thynnus*

Thunnus thynnus, which belongs to the Western Atlantic stock, migrates from the Atlantic Ocean to spawning areas in the Mediterranean Sea (Medina et al., 2002). Spawning starts in the Levantine Sea in May (Karakulak et al., 2004) and ends in July in the central and western Mediterranean (Abascal et al., 2004; Corriero et al., 2003; Susca et al., 2001; Zupa et al., 2009), when the water temperatures is 23°C approximately (Schaefer, 2001).*Thunnus thynnus* shows a particular behavior during reproduction, with a change in diving time and depth (Block et al., 2001; Teo et al., 2007). In the Atlantic spawning area, *Thunnus thynnus* exhibits deep-diving behaviors (Stokesbury et al., 2004; Teo et al., 2007) and makes oscillatory dives at night with frequent resurfacing. Similar behaviors have been observed for *Thunnus orientalis* (Kitagawa et al., 2006) and *Thunnus albacares* (Schaefer, 2001) during the reproductive season. In Mediterranean spawning areas, aggregations of *Thunnus thynnus* at different sizes and/or ages were observed. The Atlantic bluefin tuna does not exhibit sexual dimorphism and exhibits asynchronous ovarian development (Schaefer, 2001). ABFT releases oocytes under favourable environmental conditions for external fecundation and larval survival in the water column (Medina et al., 2002). This species reproduces numerous times during its life and is an iteroparous species, and both the reproductive potential and the number of eggs increase in relation to age and size (Api et al., 2018). Studies on wild bluefin tuna have estimated that the number of eggs in a single batch is approximately 93,000 per kg body weight (Medina et al., 2002).

ABFT gonads are paired, elongated organs located in the abdominal cavity and suspended by a mesogonad. The testis comprises lobules radiating from the longitudinal main sperm duct toward the periphery (Abascal et al., 2004). The testicular structure is cystic, and each cyst comprises a clone of germ cells branched by the cytoplasm of Sertoli cells. Spermatogonia were distributed throughout the seminiferous lobules, indicating that the testis was of the unrestricted spermatogonial type. According to Medina et al. (2016) and Corriero et al. (2007), testis classification is based on the following key histological features: early spermatogenesis (ES), late spermatogenesis (LS), and spent (S). The classifications based on the histological features are summarized in Table 3.

Stage	Histological features
Early spermatogenesis (ES)	Mainly cysts with spermatogonia, minimal presence of spermatocytes, spermatids and spermatozoa
Late spermatogenesis (LS)	Cysts with spermatids; lumina of the seminiferous lobules and central system of ducts filled with spermatozoa
Spent (S)	Cysts consisting mostly of spermatogonia; lumina of seminiferous lobules completely empty or showing loose residual sperm

Table 3 - Microscopic maturity classification for ABFT male according to Medina et al., (2016) and Corriero et al., (2007).

Ovaries of ABFT are hollow organs with a round cross section that join caudally in a common oviduct, which opens to the exterior in the urogenital pore. Ovaries of adult individuals consist of a thick muscle wall and ovigerous lamellae, containing oogonia and oocytes at different stages of development, embedded in a mass of connective tissue (Rooker et al., 2007). In both the testis and ovaries, perigonadal fat operates as a fat deposit and is exhausted during migration to spawning grounds to sustain gonadal development (Mourente et al., 2002). According to Medina et al. (2016), ovarian classification is based on the following key histological features: resting (R), active nonspawning (ANS), active spawning (AS), and inactive immature (IM). The classifications based on the histological features are summarized in Table 4.

Stage	Histological features
Resting (R)	Previtellogenic or early vitellogenic oocytes. No atresia
Active nonspawning (ANS)	Advanced vitellogenic oocytes and no to minor (<50%) α atresia
Active spawning (AS)	Advanced vitellogenic oocytes and no to minor (<50%) α atresia plus POFs and/or migratory-nucleus oocytes
Inactive mature (IM)	Previtellogenic or early vitellogenic oocytes plus α and/or β atresia, or advanced vitellogenic oocytes plus major (>50%) α atresia

Table 4 - Microscopic maturity classification for ABFT female according to Medina et al., (2016).

The liver: a multifunctional organ

The fish liver is located in the anterior part of the body cavity and receives blood through both the portal vein and hepatic artery (Figure 3). Most of its blood (70–80%) comes from the portal vein, which conveys blood containing nutrients absorbed in the intestine. Three forms of fish liver can be found: tri-lobed, bi-lobed or no lobulation (Buslé & Anadon, 2018). Each lobule consists of two parts: parenchyma, constituted by hepatocytes, macrophages, endothelial cells, and sinusoids, and non-parenchyma, constituted by veins, arteries, and bile ducts. The sinusoids are capillary networks that are localized in the space between the hepatic parenchyma in which the hepatocytes are arranged (Buslé and Anadon, 2018). Hepatocytes have a hexagonal shape and are distributed in tubules or cordons (Hampton et al., 1985).

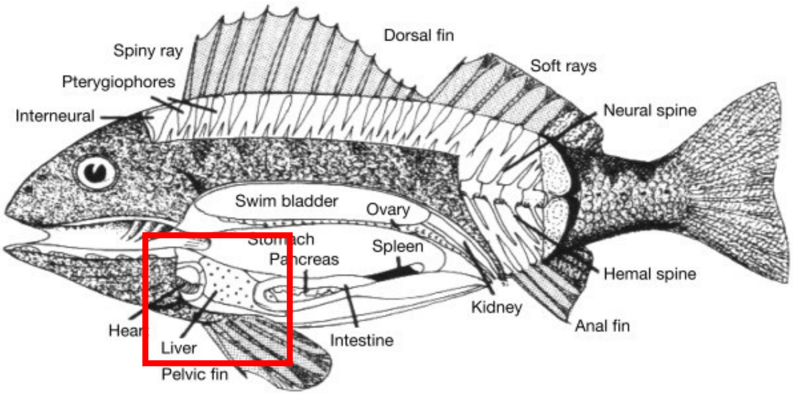

Figure 3 - Main features of a typical acanthopterygian teleost, the carp *Cyprinus carpio*. Reproduced from Bone, Q. and Marshall, N.B. (1995).

Liver plays an important role in several biological processes. As mentioned above, liver is directly involved in reproduction, by producing vitellogenin and ZPs proteins. Furthermore, during reproduction, lipids in the liver are mobilized towards the ovary in the form of neutral lipids, lipoproteins, and triglycerides. In fact, during the reproductive season, the lipid content in the liver of mature females decreases significantly, especially in capital breeder species, whereas during the no reproductive season, females store lipids in the liver, muscle, and ovary to use them in the next reproductive season (Mourente et al., 2002; Sardenne et al., 2022; Wiegand, 1996; Zudaire et al., 2014).

The liver is also involved in metabolism, both anabolism (synthesis) and catabolism (degradation, with the release of energy) (Buslé and Anadon, 2018). For the lipid metabolism, the liver synthesizes triglycerides, fatty acids, cholesterol, phospholipids and lipoproteins, also intervening in the synthesis of different plasma proteins. Lipids, amphipathic molecules that are insoluble in water, are an important energy resource for many biological processes. They can be assimilated through feeding and accumulate as energy reserves. They can also be synthesized at the hepatic level and used as membrane organelles. Lipid transport to different organs is mediated by lipoproteins (Tocher, 2003). Lipoproteins vary in size, structure, protein:lipid ratios, and relative proportions of different lipid classes, leading to density differences that are used to separate and classify the different types. There are different types of lipoproteins, including high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL), which are characterized by different lipid contents. Furthermore, the lipid composition of lipoproteins varies among different fish species. Therefore, the proportion of total lipids decreases from VLDL to LDL and HDL (Babin & Vernier, 1989; Babin, 1987).

Lipogenesis is the term used to describe the biosynthetic reactions for the formation of new endogenous lipids. The ultimate carbon source for the biosynthesis of new lipids is acetyl-CoA, which is formed in the mitochondria by the oxidative decarboxylation of pyruvate (carbohydrate source) or oxidative degradation of some amino acids (protein source). Lipogenesis is catalyzed by the cytosolic fatty acid synthetase (FAS) multienzyme complex and is characteristic of vertebrates (Sargent et al., 2003). The main products of FAS are the saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid). In the case of marine fish, saturated fatty acids are accumulated through the diets rich in lipid, especially by top predator marine fish that are not likely to synthetize the saturated fatty acids de novo (Regost et al., 2001). Saturated fatty acids can undergo desaturation and elongation to be further transformed into long-chain polyunsaturated fatty acids (LC-PUFA). LC-PUFA are necessary for correct organism function, promoting fecundity, egg hatching and viability, larval survival, and somatic growth and movement (Koven et al., 2018; Lubzens et al., 2017; Rainuzzo et al., 1997).

The degradation of fatty acids (β-oxidation) takes place in two different organelles, mitochondria and peroxisomes. Fish capacity to catabolize fatty acids depends on several factors such as fish size, life stage, and season (Leaver et al., 2008). Mitochondrial β-oxidation is mediated by a carnitine-dependent transfer of acyl groups across the inner mitochondrial membrane. Carnitine palmitoyltransferase-1 (CPT1) in the outer mitochondria membrane converts acyl-CoA to acylcarnitine, which is then transported over the inner mitochondrial membrane in exchange with a free carnitine molecule. In the mitochondrial matrix, carnitine palmitoyltransferase-2 (CPT2) catalyses the reversible transfer of acyl residues between carnitine and CoA to form acyl-CoA thioesters that are then β-oxidised (Turchini & Francis, 2009). Peroxisomal β-oxidation resembles mitochondrial β-oxidation, but with a different enzyme and represents a less efficient process from an energetic point of view because half of the energy produced is lost in the form of heat. In fish, the contribution to the total β-oxidation capacity of both organelles depends on the tissue, and peroxisomal β-oxidation is a relevant pathway of fatty acid degradation in the fish liver (Nanton et al., 2003; Stubhaug et al., 2005). Among the molecular pathways involved in the regulation, storage, transport, and synthesis of lipids in the liver, the most important is *Ppar signaling* pathway.

The peroxisome proliferator-activated receptors (PPARs) are a nuclear receptor that dimerize with retinoid X receptor (RXR) and bind to PPAR-responsive DNA regulatory elements. PPARα and β regulate FAβ-oxidation and play key roles in glucose storage, lipoprotein capture, and inflammation reduction (Serhan, 2014), while the activation of PPARγ orchestrates adipocyte function and differentiation as well as lipid storage within adipocytes (Varga et al., 2011). PPARα is abundantly expressed in the liver, whereas PPARβ and PPARγ are expressed at lower levels. PPARα, being the major regulator of the hepatic response to fasting, induces the expression of a variety of genes involved in FA catabolism and ketogenesis. This pathway regulates the expression of a multitude of genes involved in lipid metabolism and includes genes such as *Fatty-acid-binding synthesis proteins* (FABP) and the gene involved in the synthesis of lipoprotein lipase (*LPL*). The *PPAR signaling* pathway regulates carnitine palmitoyltransferase-1 (CPT1) synthesis. Cholesterol-sensitive factors (SREBPs), which are involved in lipid metabolism, are responsible for the expression of genes fundamental to the synthesis and absorption of lipids (Maradonna & Carnevali, 2018).

In addition to the lipid metabolism, the liver plays an important role in the metabolism of xenobiotics and immune response. The integrity of the aquatic environment, in this case marine environment, and its biodiversity have long been threatened by the presence of persistent, toxic, and bioaccumulable pollutants. Among these compounds are heavy metals, carried mainly by industrial discharges, inorganic pesticides and fertilizers, anthropogenic activities, and atmospheric deposits. The accumulation of toxic compounds in fish depends on numerous factors such as the physiological rates of absorption and elimination (Biller-Takahashi & Urbinati, 2014). Therefore, the liver is constantly exposed to a wide variety of bacteria, environmental toxins, and food antigens. To efficiently and rapidly protect against these potentially toxic agents, the liver depends on its strong innate immune system, which acts as an important innate immune organ (Racanelli and Rehermann, 2006). The innate response represents the first line of response against a wide spectrum of possible external agents. Innate immunity receptors, PRRs (Pattern Recognition Receptors), play a key role in the immune system. Intracellular signalling pathways activated by PPRs, such as NF-kB (Nuclear factor-κB) and IRF3 (Interferon Regulatory Factor 3), induce the production of molecules, such as inflammatory cytokines, involved in the response to any harmful substances, inorganic and organic and/or pathogen ingress. In fish, hepatocytes are responsible for the production of 80–90% of the circulating innate immunity proteins in the body, including proteins of the complement system and acute phase proteins of the inflammatory response (cytokines, interleukins, chemokines) (Jensen et al., 1997; Roy et al., 2017). However, adaptive immunity is stimulated by exposure to external agents. The main characteristics of adaptive immunity are high specificity of antibodies and their ability to memorize exposure to the same pathogen. The adaptive immune response can be classified into two types depending on the components of the immune system: humoral immunity, mediated by B lymphocytic cells, and cellular immunity, mediated by T lymphocytes. B cells in fish produce immunoglobulin (Ig) on their surface and secrete antibodies specific to antigens (Fillatreau et al., 2013). Two classes of immunoglobulins, IgM and IgD, are known to be present in fish. However, in 2005, a third class of immunoglobulins in teleosts, IgT/IgZ, was found to be involved in the immune response in the gastrointestinal mucosa (Salinas et al., 2011). T cells include both natural killer (NK) lymphocytes and T cells, the NK lymphocytes react spontaneously and immediately before T cells (Nakanishi et al., 2015). Furthermore, the complement system of fish is considered more effective than that of mammals and is one of the most important innate compounds for host protection because of the production of inflammatory mediators (Boshra et al., 2006; Nonaka et al., 1981). The complement system consists of soluble and membrane proteins in an inactive form or in low levels of spontaneous activation, which are triggered by a sequential pathway, since the initial stimulus contributes to the proteolysis of the next component. Activation can be triggered by three pathways: i) the classical pathway, an antibody-dependent activation by antigen-antibody complexes, ii) an alternative pathway, prompted by microorganisms or antigen-antibody complexes, and iii) the lectin pathway, triggered by bacterial surface carbohydrates. The alternative pathway is highly efficient for innate recognition and is considered the most important among the three activation pathways. It can be easily triggered by various gram-negative bacteria and lipopolysaccharides and can cause cytolysis. Alternatively, the classic pathway performs interaction between innate and specific systems (Boltaña et al., 2011; Boshra et al., 2006; Holland & Lambris, 2002). Two components of the complement system, C5a and C3b, play a central role in the recruitment of phagocytes and inflammation. C5a and C3b are chemotaxis proteins for neutrophils and macrophages because they remain linked to the bacterial wall, triggering biological processes such as opsonization, phagocytosis, chemotaxis of leukocytes, and inactivation of released bacterial toxins. The complement system is widely used as an indicator of the immune status because of its contribution to host protection. The function of the complement system in cellular activation, phagocytosis, chemotaxis, inflammatory reactions, and lysis of pathogen cells is well known mainly for its ability to destroy pathogens through membrane injuries, commonly characterized by pores (Bayne & Gerwick, 2001; Boshra et al., 2006; Ellis, 2001; Secombes, 1996).

The liver plays an important role in the detoxification process. In the liver, both hepatocytes and macrophages contribute to detoxification processes. For example, the cytochrome P450 (CYP or P450) system comprises a large superfamily of heme proteins that are involved in the oxidative metabolism of numerous lipophilic exogenous and endogenous substrates, including drugs, aromatic hydrocarbons, pesticides, fatty acids, prostaglandins, and steroids (Goksøyr, 1995; Shoieb et al., 2020; Stegeman et al., 1997). CYPs gene expression is controlled by nuclear receptors. The modulation of the CYP profile is often used as an indicator of exposure to environmental contaminants. CYP1A induction, specifically, is commonly used to identify the presence of polyaromatic hydrocarbons (PAHs) and other aryl hydrocarbon receptor (AhR) agonists, the specific nuclear receptors responsible for the induction of CYP1 genes in all vertebrates (Hahn, 1998). The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BaP), and certain non- and mono-ortho-substituted polychlorinated biphenyls (PCBs) can induce the transcription of CYP1A genes through the aryl hydrocarbon (Ah) receptor. The AhR receptor is responsible for activating the CYP signalling pathway (Hahn, 1998). Several authors (Gräns et al., 2010; Labrecque et al., 2012; Matthews & Gustafsson, 2006; Mortensen & Arukwe, 2007) have suggested that Ahr and ERs compete for the same cofactor (Arnt2), which can bind both the xenobiotic response element (XRE) and estrogen response element (ERE) domains. Binding to ERE inhibits the activation of P450 signalling pathways. Therefore, cytochrome P450 enzymes (CYPs) are often used as indicators of exposure to environmental contaminants. In addition to CYPs activity in hepatocytes, phagocytic cells have also been found in the liver. Melanomacrophages (MMs) are phagocytic pigment-containing cells principally located within the stroma of the spleen, kidney, and liver. There have also been reports of their occasional occurrence in the gills, brain, and gonads (Macchi et al. 1992).

MMs circulate freely in blood vessels, pass through the intact vessel wall (margination and diapedesis mechanisms), and migrate in the parenchyma of the liver, spleen, and/or kidney (Agius, 1980; Passantino et al., 2014). In the parenchyma, MMs can be single or form aggregates or centers (MMCs) (Agius, 1980; Imai et al., 2022; Saunders et al., 2010). Furthermore, MMCs contain three different pigments (hemosiderin, lipofuscin, and melanin) (Agius & Roberts, 2003). Hemosiderin, which appears brown, can be found during the degradation and phagocytosis of erythrocytes, followed by iron, which is carried out by melanomacrophages (Agius and Roberts, 2003). Lipofuscin, which appears yellow-brown, can be found in MMCs during the beta-oxidation of fatty acids due to pollutants or aging of animals (Imai et al., 2022). Melanin, which appears dark brown, is also involved in the neutralization of the free radicals released during cell membrane rupture (Qualhato et al. 2018). Their presence may be an important pathological indicator (Dang et al., 2019). MMs play an important role in the destruction, detoxification, and recycling of endogenous and exogenous materials, such as erythrocytes, nanoparticles, parasites, and xenobiotics (Basilone et al., 2018; Corriero et al., 2013; Dang et al., 2019; Fishelson, 2006; Haaparanta et al., 1996; Mela et al., 2007; Nowak et al., 2021; Passantino et al., 2014; Passantino et al., 2020; Qualhato et al., 2018; Stosik et al., 2019; Vigliano et al., 2006; Widdicombe et al., 2020). For example, in Southern Bluefin Tuna (*Thunnus maccoyi*), the area of melanomacrophage centers (MMCs) increased with increasing intensity of *Cardicola* sp. infection, and the authors suggest that the infection and MMCs have the potential to be used as an indicator to assess the health effects of parasites on fish (Widdicombe et al., 2020). In *Clarias gariepinus*, the authors examined the potential histopathological effects of 4-nonylphenol on the hepatic MMCs. We found that the size and number of MMCs were dramatically higher in the livers of 4-NP-exposed fish than in the control fish, suggesting the utility of MMCs as a cytological biomarker for aquatic exposure to 4-NP (Sayed et al., 2019). Finally, in the European anchovy (*Engralius encrasicolus*), the authors verified the suitability of using MMCs as response biomarkers of marine pollution in European anchovy from four areas. Anchovies sampled from three areas exposed to pollutants of industrial/agricultural origin showed areas and numbers of MMCs significantly higher than those in anchovies from a marine area not subjected to any source of pollution. The number, size, and distribution of MMs and MMCs vary according to age, nutritional level, and stress conditions (Fishelson, 2006). MMCs are encapsulated by a collagen capsule or not, and the color and form changes in relation to the species in which they are formed (Figure 2). Thus, MMs are considered environmental biomarkers in the liver (Carreras-Colom et al., 2022; Nowak et al., 2021; Passantino et al., 2020; Viana, 2021). In particular, an increase in MMs density in *Thunnus thynnus*, *Engraulis encrasicolus*, *and Clarias gariepinus* was observed after exposure to endocrine destroyers, toxic components, and xenobiotics (Agius & Roberts, 2003; Basilone et al., 2018; Passantino et al., 2014; Passantino et al., 2020; Sayed et al., 2019). MMCs are associated with or incorporated within blood vessels, bile ducts, or necrotic areas (Pronina et al., 2014) (Figure 2). In addition, in teleosts, melanomacrophage centers play an important role in the immune response and immunological protection from different types of stress, including exposure to heavy metals, free radicals, herbicides, or parasitic infections. Therefore, they are used as biomarkers to assess the health status of fish (Maria et al., 2020; Qualhato et al., 2018; Sayed et al., 2019). Lymphocytes, granulocytes, and other immune cells can be found in association with MMCs. Ellis and de Sousa (1974) indicated, for the first time, the role of MMs and MMCs in the development of defence reactions in fish. These authors identified MMCs as the likely place where the antigen, being taken up by MMs, can react with the immune system units, lymphocytes, in which signals initiating specific immunity are generated. Retention of the antigen in the MMCs, and, in further stages, the activation of macrophages and lymphocytes, the synthesis and secretion of antibodies and the development of immunological memory are confirmed by Press et al., (1996) in their studies on Atlantic salmon (*Salmo salar*) and by Zapata (2022) in jawed fish.

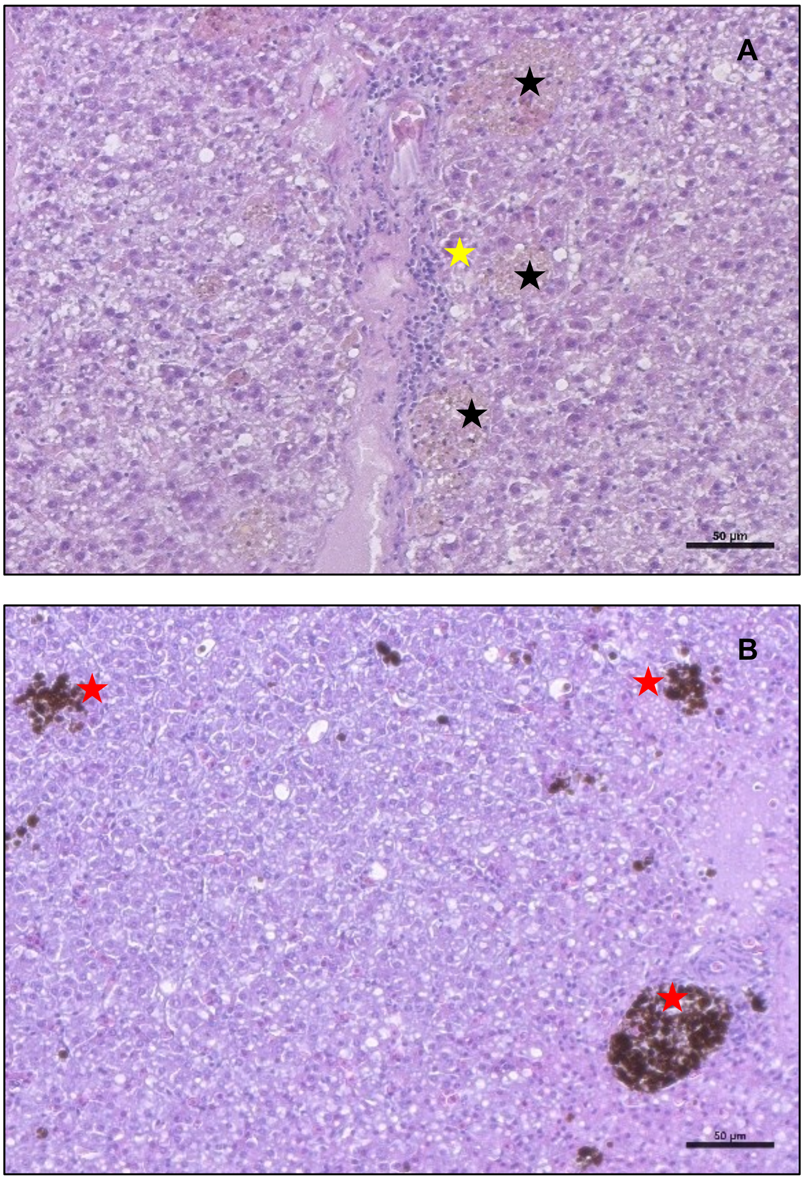

Figure 2 - Histological feature of melanomacrophages centres (MMCs); A) MMCs (black stars) in the liver of *Thunnus thynnus* associated to blood vessels, with the presence of lymphocytes (yellow star); B) MMCs (red stars) in the liver of *Xiphias gladius* associated to blood vessels.

The Omics

The development of *high-throughput technologies*, particularly omics (genomics, transcriptomics, proteomics, and metabolomics), has provided deeper insight into the biology of organisms and their interaction with the environment. Various advances at different biological levels have revolutionized our ability to analyze genome structure and function (Del Giacco & Cattaneo, 2012): (1) at the level of DNA, including the development of high-throughput DNA sequencing and next-generation sequencing technologies (NGS); (2) at the level of gene expression ("transcriptomics") using microarray or RNA sequencing; (3) At the level of protein products, with improved analysis of proteins ("proteomics") using tandem mass spectrometry and (4) At the metabolic level, with the detailed analysis of low-molecular-weight cellular constituents ("metabolomics") (Aizat et al., 2018). The term **omics** refers to a field of study in the biological sciences that ends with -omics (gen**-omics**, transcript**-omics**, prote**-omics**, or metabol**-omics)**. These omics approaches are now increasingly popular, as evidenced by the growing number of publications throughout the years (https://www.omicsdi.org/). Genomics studies the structure, function, evolution, and mapping of genomes and aims to characterize and quantify genes. Transcriptomics is the quantification and characterization of all RNA transcripts in a cell, tissue, or organism (Kim, 2016). Furthermore, in the fisheries context, the analysis of Single Nuclear Polymorphism (SNP), called ddRAD sequencing, is used to define the population structure of stock and improve the knowledge of the interspecific and among population differences (Kai et al., 2014).

Genomics

Genomics is defined as the study of the structure, function, and diversity of a genome. The aim of genomics is to extend analyses originally limited to one or a small number of genes, to large numbers of genes, or to the complete genome. Initial efforts in genomics have been directed toward humans and mammals as biomedical model organisms. In recent years, this has been extended to a wide range of fish species, particularly teleosts, which occupy diverse environments within aquatic ecosystems. The assembly of genomes of several teleost species, such as *Seriola dumerii* (Araki et al., 2018) and *Salmo salar* (Davidson et al., 2010), has been reported. The application of genomic approaches in fisheries management can improve our knowledge of reproduction, growth, and immune responses of fish, including the evaluation of stock diversity (Ward, 2000). Sequencing procedures are better merged with data acquisition and analysis, moving away from low-efficiency historical Sanger sequencing approaches and facilitating an extraordinary increase in data output (Aizat et al., 2018). Two new genomic approaches have been developed to facilitate genome study. The first is a second-generation approach, such as Illumina, which generally starts with DNA fragmentation, DNA end-repair, adapter ligation, surface attachment, and in-situ amplification. These "*short-read*" sequencing technologies involve the parallel sequencing of short reads. However, owing to the nature of short-read technologies, sequencing data over long stretches of DNA must be reassembled, presenting disadvantages in detecting structural variations. The second is third-generation sequencing, such as Oxford Nanopore technology, which can achieve read lengths of 10 kb, much greater than Sanger or short-read sequencing technologies (Stefan et al., 2022). These "*long-read*" technologies can overcome issues encountered with short-reads, such as structural variant detection. A limitation of these third-generation technologies compared to second-generation methods is the accuracy of the reads, which continues to improve over time, particularly with new software analyses (Stefan et al., 2022). Both technologies can be combined to produce a high-quality genome, called '*hybrid assembly*, in which the read data produced from two different sequencing platforms, Illumina (*short read*s) and Oxford Nanopore Technologies (*long reads*), are used. Genomics can be applied to fisheries and their management (Hauser and Carvalho, 2008). The fundamental purpose of fisheries management is to ensure the sustainability of fish stocks through regulatory and enhancement actions that promote the economic and social well-being of fishermen and industries (Hilborn et al., 2003). Genomic tools provide an unprecedented opportunity to contribute to fisheries management by providing insights into the evolutionary forces driving the spatial and temporal heterogeneity of stocks (Hilborn et al., 2003). Furthermore, genomics can help address fundamental questions in fisheries management such as stock identification, population structure, and adaptive response of fishes to environmental change (Bernatchez et al., 2017; Dudgeon et al., 2012)

Transcriptomics

The transcriptome is the set of transcripts in a cell that is specific to the developmental stage or physiological conditions. The study of transcriptomes is important to understand the functional elements of the genome, to unveil the genes involved in several biological processes, and to highlight the molecular processes that occur in cells or tissues. The aims of transcriptomics are to i) catalogue all transcripts (mRNA, non-coding RNA, transfer RNA, messenger RNA), ii) determine the transcriptional structure of genes (5′ and 3′ ends, splicing patterns, and other post-transcriptional modifications), and iii) quantify the expression levels of transcripts (Wang et al., 2009). Next-generation RNA sequencing (RNA-seq) is a high-throughput experimental technique that allows the sequencing of cDNA with very high redundancy (depth). This technique can provide quantification-by-sequencing of RNA abundance and analysis of RNA sequence variation in biological samples (Hrdlickova et al., 2017). RNA transcript analysis can be both qualitative (allowing the assembly of transcriptomes from non-model species) and quantitative (providing a measure of the relative quantities of different transcripts in a sample). Three steps are necessary to sequence the transcriptome of a biological sample:1) the RNA has to be purified, 2) an RNA (or cDNA) library needs to be synthesized, and 3) eventually the library is sequenced, undergoing high-speed sequencing to produce millions to billions of short "*reads*" (Cellerino & Sanguanini, 2018). The reads measure approximately 30-400 bp, depending on the sequencing technology used and the size of the transcript (Wang et al., 2009). Given a read, the best sequence match must be found (i.e., to align with the highest possible accuracy) in the reference genome (or transcriptome). This process is known as *mapping*. In the case of a comparative analysis between two different transcriptomes, differentially expressed genes (DEGs) were detected. A gene was considered differentially expressed if the p-value was smaller than an arbitrary significance threshold. The most widely used is the false discovery rate (FDR), where the significance threshold is a minor of 0.05 (Li et al., 2012). A method to analyze the big data of the transcriptome is to understand gene functions and their categories. This method is widely used to understand that some DEGs could belong to different "gene categories" It is also possible to verify, by applying enrichment analysis, whether a gene category (rather that individual genes) is significantly up- or down-regulated according to a given condition (Marguerat & Bähler, 2009). GO analysis is a useful practice for understanding the biological sense of a long list of DEGs. The Gene Ontology (GO) is a precise vocabulary composed of defined phrases, called GO terms, that describe the molecular actions of gene products, the biological processes in which occur and their cellular locations. The aim of the Gene Ontology (GO) Project (Harris et al., 2006) is to1) maintain and develop its controlled vocabulary of gene and gene product attributes; 2) annotate genes and gene products, and assimilate and disseminate annotation data; and 3) provide tools for easy access to all aspects of the data provided by the project, and to enable functional interpretation of experimental data using GO, for example via enrichment analysis (Dessimoz & Walker, 2017). GO annotation has three independent roots, called domains, which represent three different aspects of the biological properties associated with a gene product: Cellular Components (related to the localization of the gene product in the morphology of the cell), molecular functions (describing activities that occur at the molecular level), and Biological Processes (involved in processes and pathways) (Dessimoz & Walker, 2017). Another way of clustering genes is to group them based on their involvement in each pathway. This approach was adopted by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000), which makes it possible to visualize up- and downregulated genes within a pathway coloring each gene according to its value of differential expression. KEGG pathway analysis offers a clear description of complex transcriptional processes. In the KEGG database, each gene is annotated with one or more unique pathway identifiers. The main disadvantage of using KEGG as a reference is that most genes are not mapped to any KEGG pathway, especially in non-model species. A combination of GO and KEGG analyses can elucidate molecular, cellular, and biological processes with a wide view, overcoming the disadvantages of the two analyses. Recently, fisheries omics has become an emerging field that applies genomic and transcriptomic tools to solve questions related to fisheries management. RNA-seq has been widely applied in several studies for the conservation and preservation of fisheries resources, investigating genes involved in adaptation to environmental changes, and in response to overfishing (Machado et al., 2018, 2020; Sun et al., 2016; Valenzuela-Quiñonez, 2016).

Population genetics: The double digest RAD sequencing

The study of genetic variation within populations and allele frequencies in populations through time and space is the main subject of population genetics. Genetic variation permits species and populations to adapt to their environment. New genetic variation arises in a population from either spontaneous mutation of a gene or immigration of an individual, originating from a genetically different population. The number and relative abundance of alleles in a population are a measure of genetic variation (Çiftci & Okumus, 2002). Next-generation genome sequencing technologies have been developing rapidly over the past decade, providing many extraordinary tools to conduct population genetic studies of both model and non-model organisms at the whole-genome scale (Kim, 2016). A large number of single-nucleotide polymorphisms (SNPs) are easily identified at the population level (Wu et al. 2014; Xu et al. 2014). Genotyping-by-sequencing approaches have been developed to genotype thousands of SNP loci at the population level. SNPs are used on fish populations to understand their genetic diversity, population structure, demographic history, and adaptive evolution. Double-digest restriction enzyme-associated DNA sequencing (ddRAD-seq) (Peterson et al., 2012) is one of the most recently developed RAD variants, known for its relative flexibility and ease of use. In contrast to RAD-seq, in which only one restriction enzyme is used, ddRADseq produces sequencing libraries consisting of a subset of genomic restriction digest fragments generated by cuts with two restriction enzymes (i.e., having one end from each cut). Subsequently, the fragments were sequenced using next-generation sequencing technologies (for example, Illumina NovaSeq6000) to obtain SNPs located next to target restriction sites. Compared to previous methods, such as microsatellites, ddRADseq-based SNPs are more reliable for interpreting fine-scale population structure and for the analysis of population genetic variance, even with a small number of samples (Liu et al., 2019). Using this method, genetic and fishery management can interact (Bagley et al., 2021; Çiftci & Okumus, 2002; Le Moan et al., 2019; Liu et al., 2019; Maroso et al., 2018; Nyinondi et al., 2020; Puncher et al., 2021; Puritz et al., 2016; Saha et al., 2021; Zhao et al., 2018).

Genetic variation (i.e., genomic variation) can be measured using several methods. One useful measure of genetic diversity is population heterozygosity (H), which is defined as the mean percentage of heterozygous loci per individual (or equivalently, the mean percentage of individuals heterozygous per locus). Heterozygosity can be classified into observed (H_o) and expected (H_e). While expected heterozygosity is estimated from allele frequencies, observed heterozygosity is estimated directly from individual genotypes and depends on both the amount of genetic variation in the population and the level of inbreeding, which increases homozygosity. Heterozygosity must be analyzed with a high number of SNPs and samples to avoid incorrect estimations (Schmidt et al., 2021). Furthermore, one of the most commonly used metrics to study population structure is the F-statistic, developed by Wright (1965). F-statistics divides the observed genetic variability into two main components: the inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). The inbreeding coefficient (F_{IS}) characterizes the probability of two alleles from any random individual in a population that is inherited from the same ancestor (i.e., inbreeding). It provides insights into the reproductive dynamics of a local population and the breeding of consanguineous populations (Ouédraogo et al., 2021). The fixation index (F_{ST}) is a measure of population differentiation owing to its genetic structure. It is frequently estimated from genetic polymorphism data such as single-nucleotide polymorphisms (SNP) or microsatellites. It is one of the most widely used statistics in population genetics (Wright, 1965). To demonstrate the level of genetic similarity or genetic distance between two populations, Nei’s distance is one of the most frequently used measures of genetic divergence between species or between populations within a species, measuring the distance from a common ancestor. Populations with many similar alleles had small genetic distances. This indicates that they are closely related and that they have a recent common ancestor (Çiftci & Okumus, 2002; Nei et al., 1976; Nei & Tajimat, 1987). The last useful measure of genetic variance is the allelic richness. Allelic richness is defined as the total number of alleles in a population and is indicative of a population's long-term potential for adaptability and persistence (Allendorf García-Dorado, 2013; Greenbaum et al. 2014, Allendorf et al., 2012). Allelic richness is more susceptible to population bottlenecks (Leberg, 2002; Luikart et al., 1998); therefore, it is worthwhile to examine long-term responses to natural and artificial selection (Caballero & García-Dorado, 2013; Hill & Rasbash, 1986; James, 1971; Medugorac et al., 2011; Vilas et al., 2015; Wilson et al., 2009). There is an indication that high allelic richness increases adaptability by making a larger portion of the genome accessible through fewer mutational events (Wagner et al., 2008). This measure is of key importance in the conservation and management of fish stocks (Foulley and Ollivier, 2006). The loss of allelic diversity can have harmful effects in marine populations where the loss of heterozygosity is minimal (Ryman et al. 1995b). Thus, reducing the population size from millions to thousands could have no effect on heterozygosity, but could result in a decline in allelic richness. This reduction could have several consequences, such as inbreeding depression that might limit population growth and the ability of the population to evolve in a new or changing environment (Allendorf et al., 2012). Overfishing is one of the most significant causes of genetic erosion and loss of genetic diversity in a variety of commercial fish species (Allendorf et al., 2014; Pinsky & Palumbi, 2014).

Finally, the coupling of ddRAD sequencing with a genome assembly can highlight the complex genetic structure between populations and characterize the genetic diversity among fish stocks at the chromosome level, especially genes (De Wit et al., 2015; Vandeputte et al., 2019; Vendrami et al., 2019). Indeed, these genomic approaches are considered more suitable than classical neutral markers (e.g., microsatellites) for investigating functional genetic variation and fitness traits in the context of conservation biology (Ouborg et al., 2010).

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CHAPTER 1

A window of vulnerability: chronic environmental stress does not impair reproduction in the swordfish *Xiphias gladius*

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1.1 Abstract

Xiphias gladius is an important fishing resource. The Mediterranean stock is affected by overfishing and is declining. In this light, the aim of this study was to evaluate the cross-talk among metabolism, stress response, immune system and reproduction in immature and mature females, coupling histological and transcriptomic approaches. The transcriptome of livers from 3 immature and 3 mature females was analyzed using the Artificial Intelligence RNA-Seq. For the histological analysis, ovary and liver samples were collected from 50 specimens caught during the reproductive season in the Mediterranean Sea. A total of 750 genes were differentially expressed between the livers. The gene ontology analysis showed 91 upregulated and 161 downregulated biological process GO terms. Instead, the KEGG enrichment analysis revealed 15 enriched pathways. Furthermore, the binding occurring between estrogen receptors and aryl hydrocarbon receptor nuclear translocator, upregulated in mature females, could be liable for the inhibition of detoxification pathway. Indeed, at the histological level, mature females showed a higher density and number of melanomacrophage centers, biomarkers of stress. The present findings reveal the cross-talk among response to environmental stressors, metabolism and reproduction, highlighting that mature females invest a lot of energy in reproduction instead of immune response and detoxification.

1.2 Introduction

The swordfish (*Xiphias gladius*) is a cosmopolitan, highly migratory teleost species and an important fishing resource. The Mediterranean stock has been affected by overfishing and has declined since the 1980s (ICCAT, 2016). A recovery plan was established by the International Commission for the Conservation of the Atlantic Tunas (ICCAT), including measures such as fishing fleet capacity limitations, closed fishing season and a minimum size (ICCAT, 2016). Recently, more attempts to better understand the reproductive biology of this fish (i.e., spawning area and period) and sexual maturity were carried out with promising results (Abid et al., 2019; Arocha, 2002; Gioacchini et al., 2019; Gomez Vives et al., 2005; Marisaldi et al., 2020). In this context, deepening the knowledge about metabolic requirements during reproduction, growth pattern and responses to external stimuli are central points. Thus, a deep knowledge at the molecular level of the genes underlying these processes is necessary to obtain a more complete picture of the reproductive potentiality of this species. Recently, RNA-seq was widely applied in several studies for the conservation and preservation of the fisheries resource, investigating genes involved in adaptation to environmental changes (Machado et al., 2020; Valenzuela-Quiñonez, 2016). Notably, swordfish ovarian and liver transcriptome analysis was performed (Basili et al., 2021; Gioacchini et al., 2019). In particular, the transcriptome of ovaries from mature and immature females, caught in the breeding season, were investigated in order to identify and characterize the molecular network involved in sexual maturity and in the circadian rhythm. Despite these papers focusing attention on the genes involved in sexual maturation and reproduction such as estrogen receptor α (esrα) and three forms of vitellogenin, little attention was paid to genes involved in metabolic processes and responses to environmental stimuli. How the limited internal resources of any organism flow among reproduction, growth and response to environmental stress is a key determinant of the success of survival of the species (Harshman & Zera, 2007; Lochmiller & Deerenberg, 2000). In wild populations, this energetic balance is challenged by chronic and acute environmental stress such as chemical pollution (Thompson et al., 2009), habitat modification (Fischer & Lindenmayer, 2007) and climate changes (Radchuk et al., 2019). In teleost, in fact, an important target organ of such environmental stressors is the liver, and its health status is used for biomonitoring purposes (Gernhöfer et al., 2001).The liver is responsible of several processes, such as immune response (Martin et al., 2010; Wang et al., 2019), detoxification (Shoieb et al., 2019), metabolism (Ayisi et al., 2018; Turchini et al., 2009) and reproduction (Bemanian et al., 2004; Leaños-Castañeda & van der Kraak, 2007). In the liver, the lipid metabolism is investigated to identify the genes and pathways involved in fatty acid synthesis, lipid transport and oxidation process (Araújo et al., 2018). Numerous studies were conducted to understand the lipid metabolism in fish species exposed to different diets, toxic compound or nanoplastics (Hamid et al., 2021; Lai et al., 2021; Xu et al., 2021). In addition, in fish, the hepatic lipid metabolism (synthesis and mobilization) play an important role during oogenesis, (i.e., yolk formation or oocyte lipidation) and in further larval development (Hiraoka et al., 2019; Tocher, 2003). Furthermore, the histological analysis of the liver can reveal the health status of several fishes by evaluating the occurrence of melanomacrophages (MM) an important environmental biomarker (Dang et al., 2019; Fishelson, 2006; Haaparanta et al., 1996; Mela et al., 2007; Vigliano et al., 2006), which are involved in detoxification, immune response and destruction/recycle of various exogenous and endogenous materials, such as erythrocytes (ferric ion), pathogens (Nowak et al., 2021; Widdicombe et al., 2020) and nanoparticles (Qualhato et al., 2018; Stosik et al., 2019). An increase in aggregate of melanomacrophages (MMC) or single melanomacrophage cells (MMs) density has been recorded along with expression levels increase of several genes involved in detoxification and reproduction in fishes after exposure to different stressors (Ardeshir et al., 2022; Basilone et al., 2018; Nowak et al., 2021; Passantino et al., 2014). The coupling of RNA-seq and histological approaches has been widely used to investigate the effect of pollutants at the molecular and physiological level (May et al., 2019; Limonta et al., 2019; Hook et al., 2018). In fact, changes in transcripts abundance revealed a modification of molecular signaling. However, the transcriptome changes should be linked to a physiological response because they could not reflect the real RNA translation (Hook et al., 2018).

In the present study, by transcriptomic analysis, a deeper insight into the cross-talk between reproduction, metabolism and response to environmental cues across different stages of sexual maturation was provided by the livers of female swordfish. In addition, by histological assessment of MMC and MMs density/frequency, size and lipid content, we successfully identified a variation related to sexual maturity and fish size. Taken together, these results reveal the molecular relationship between metabolism, response to environmental stressors and reproduction, highlighting that mature females invest most of their energy in reproduction instead of detoxification and immune responses.

1.3 Materials and Methods

Sampling activities

Liver samples were collected from swordfish females already caught and analyzed in previous studies using different approaches and for different purposes (Marisaldi et al., 2020; Basili et al., 2021; Gioacchini et al., 2019). Briefly, the animals were caught by commercial long-liners in the central and western Mediterranean Sea. The lower jaw to fork length (LJFL) (min = 97 cm; max = 190 cm) and total weight (TW) (min = 8 kg; max = 90 kg) were recorded for each specimen. Reproductive status of females was previously determined by histological analysis (Marisaldi et al., 2020; Basili et al., 2021; Gioacchini et al., 2019), and 22 immature and 28 mature females were included in this study. The samples were collected following the guidelines of the International Commission for the Conservation of Atlantic Tuna (ICCAT). Samples of livers (~2 cm3) were fixed in a formaldehyde-glutaraldehyde solution ($NaH_2PO_4 \cdot H_2O$ + NaOH + formaldehyde 36.5% + glutaraldehyde 25% + H_2O) and stored at 4 °C until histological analyses.

Transcriptomic analysis

In order to focus on the molecular cross-talk among reproduction, metabolism and response to environmental cues between immature and mature females, we leveraged the knowledge contained in the transcriptome recently assembled and described by Gioacchini and collaborators (Gioacchini et al., 2019). The experimental dataset (Illumina paired-end 150 bp reads) of livers from 3 immature and 3 mature females was downloaded from SwordfishOmics (http://www.swordfishomics.com, accessed on 29 May 2020). Reads mapping, using to reference the swordfish genome, and differential gene expression analysis (FDR cut-off < 0.05) were performed using the A.I.R. (Artificial Intelligence RNA-Seq) software from Sequentia Biotech (https://transcriptomics.sequentiabiotech.com/, accessed on 29 May 2020), which applies empirical Bayes estimation and exact tests based on a negative binomial model (edgeR). The immature female transcriptome was used as reference group. Gene ontology analysis was performed in the A.I.R. environment.The Gene Ontology Enrichment Analysis (GOEA), based on differentially expressed genes, was performed using the clusterProfiler package (Yu et al., 2012) in RStudio environment and the *p*-values were adjusted with the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Then, the enriched GO terms were investigated based on the research questions, analysing the enrichment score and the number of genes for each GO terms. In addition, the KEGG Enrichment Analysis and BRITE functional hierarchies analysis (A-B categories) were carried out with the clusterProfiler package (Yu et al., 2012) in the RStudio environment. The *p*-values were adjusted with the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Pathways enriched by differentially expressed genes were investigated based on the topic of interest.

Experimental Validation

Validation of five genes (*elovl6, fabp1, igf-1, ers1, sbrepb1*) was performed by means of qPCR. From samples selected for transcriptomics analysis, a total amount of 1 µg of RNA was used for cDNA synthesis, employing the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). PCRs were performed with the SYBR green method in a CFX96 Real-Time PCR system (Bio-Rad) following Gioacchini and coworkers (Gioacchini et al., 2017). Acidic ribosomal phosphoprotein P0 (*arp*) and ribosomal protein L7 (*rpl7*) were used as internal standards in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls, and no primer-dimer formations were observed in the control templates as indicated by the melting curve analysis. The data obtained were analyzed using the CFX Manager Software version 3.1 (Bio-Rad), including GeneEx Macro Conversion and GeneEx Macro files and results represented by bar-plots along with the standard error. Statistical significance was attained using a *t*-test. Specific primer pairs for target genes (*elovl6:* Fw- ATATGGCCTTGTGGCTTCC, Rv- GCCATTCTGGTGCTCCTTCT; *fabp1:* Fw- GCATGAGGGGCGGATAGGAA, Rv- AAGGTCCCAGTTACCTCCACGATA; *igf-1:* Fw- TGTAGCCACACCCTCTCACT, Rv- GGGCCATAGCCTGTTGGTT; *ers1:* Fw- GACAAACGACGAACTGGCAC, Rv- CTCCCATCCTGAAGGAGCAC; *sbrepb1:* Fw- CCTGTCTAAAGGCCCTCGGT, Rv- TTAGCAGAGACCACAACGCA; *arp*: Fw- ACAGCCCAGTCTTTCCACAG; Rv- TTTAAGGTCCGGGCAACCTG; *rpl7*: Fw- GTACTGCTCGCAAAGTGGGA, Rv- GACTTTGGGGCTGACACCAT) were designed with Primer-Blast.

Histological analysis

Liver and ovary samples were serially dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Sections of 4 µm were cut with a microtome (model RM2125 RTS; Leica Biosystems, Wetzlar, Germany), stained with Mayer's haematoxylin/eosin and examined under a microscope (Axio Imager 2; Zeiss, Oberkochen, Germany). Quantification of MMCs and MMs was performed in 5 sections taken with a 20x objective (digital field area = 149,738 μm^2) while the quantification of lipids was performed in 5 sections with a 40x objective (digital field area= 39,533 μm^2) (Imai et al., 2022). The separation between each section was 40 µm. The density of MMCs and their number per mm2 of hepatic parenchyma as well as the density of lipids were measured using Fiji (Schindelin et al., 2012). The density of MMCs, MMs and the density of lipids were expressed as $\mu m^2/mm^2$. Data were first checked for normality with the Shapiro test and a Pearson’s correlation test was performed among the density and number of MMs and MMCs, the density of lipids and TW and LJFL (kg and cm, respectively) in R-studio environment using cor.test command. The *t*-test was performed between the immature and mature groups for each variable using GraphPad Prism 6 version 6.00 for Windows (GraphPad Software, La Jolla California USA).

1.4 Results

Transcriptomic analysis

In order to investigate the dynamics occurring in swordfish among immune system, metabolism, reproduction and stress response during the breeding season, the transcriptomes between mature and immature livers assembled by Gioacchini and collaborators were analyzed using a different protocol of statistical analysis and the reference genome (Gioacchini et al., 2019).

DEGs

To deepen the understanding of molecular cross-talk among reproduction, metabolism, immune system and stress response, the focus was placed on DEGs. In particular, twenty-one upregulated and twenty-one downregulated genes were identified for each process of interest (Figure 1). Five upregulated genes, including aryl hydrocarbon receptor nuclear translocator-like 2 (*arnt2*), and four downregulated genes, including cytochrome P450 family 1 subfamily A polypeptide 1 (*cyp1a1*), hepatocyte nuclear factor 4 alpha (*hnf4a*) and glutathione peroxidase (*gpx*), are involved in stress response. Five upregulated genes, including B-cell receptor CD22 B-lymphocyte cell adhesion molecule (*cd22*) and immunoglobulin superfamily member 8 (*igsf8*), and eight downregulated genes, including complement component 8, beta polypeptide (*c8b*), complement component pro-C3 (*c3*) and complement factor H (*cfh*), are involved in the immune system. Eight upregulated genes, including fatty acid-binding protein liver-type (f*abp1*) phospholipid-transporting ATPase (*drs2*), and eight downregulated genes, including elongation of very-long-chain fatty acids protein 6 (*elovl6*) and preproinsulin-growth factor I (*igf1*), are involved in metabolism. Three upregulated genes, including estrogen receptor alpha short form (*esr1*), and one downregulated gene, estrogen receptor beta (*esr2*), are involved in reproduction. Five of the DEGs identified were quantified by qPCR, and the differences were validated (Figure 2).

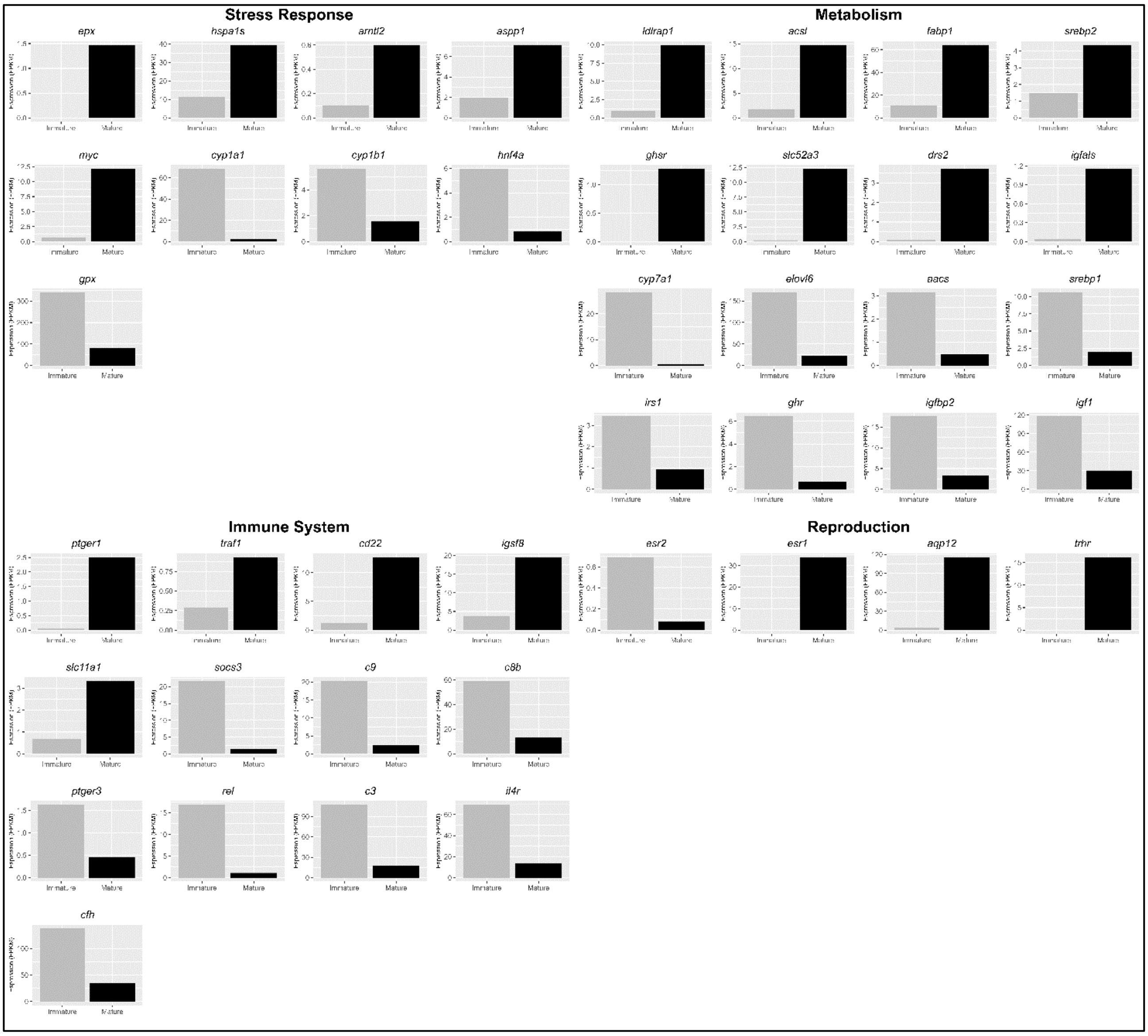

Figure 1 - DEGs analysis between livers of immature (*n*= 3) and mature (*n* = 3) females. Bar plot shows gene expression levels (FPKM) of genes involved in metabolism, immune system, reproduction and stress response. *y*-axis indicates the FPKM level.

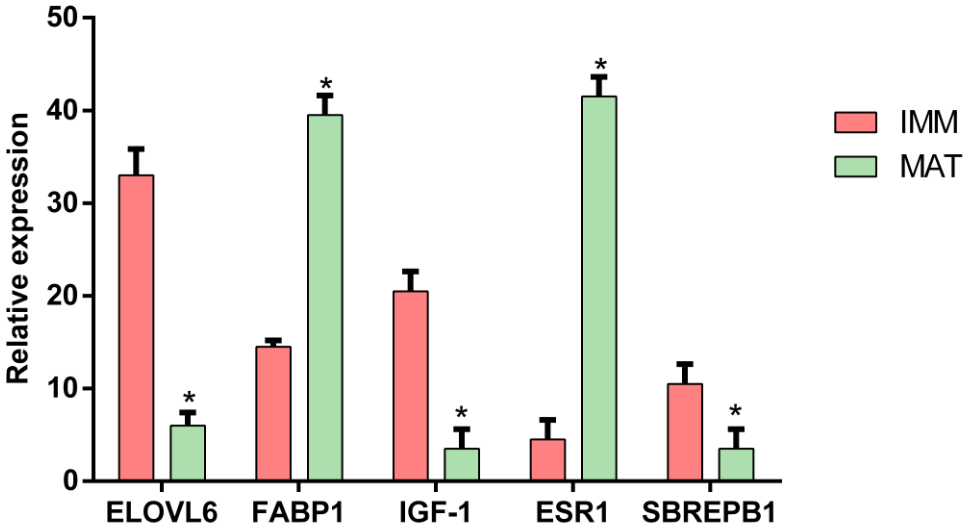

Figure 2 - qPCR validation. Bar plot shows gene expression levels of five genes, measured by qPCR between livers of immature (*n* = 3) and mature (*n* = 3) females. Significance between mature and immature for each gene is shown by an asterisk, and standard error is reported with error bars.

Gene Ontology

Using the reference genome of swordfish and the empirical Bayes estimation and exact tests based on a negative binomial model, a total of 750 differentially expressed genes (DEGs) were identified between immature and mature females: 355 upregulated and 395 downregulated. The GOEA (gene ontology enrichment analysis) identified fourteen enriched biological processes, including lipid transport (GO:0006869), response to estradiol (GO:0032355), response to bacterium (GO:0009617), egg coat formation (GO:0035803) and response to polycyclic arene (GO:1903165) (Figure 3).

The gene ontology analysis showed 91 upregulated and 161 downregulated bio-logical process GO terms. Twenty-eight upregulated and twenty-nine downregulated biological process GO terms were examined based on their involvement in the immune system, metabolism, reproduction and stress response. The livers from mature females showed upregulated GO terms such as response to polycyclic arene (GO:1903165) in-volved in stress response, response to bacterium (GO:0009617) involved in immune system, long-chain fatty acid transport (GO:0015909) involved in metabolism and response to estradiol (GO:0032355) involved in reproduction, and downregulated GO terms such as xenobiotic metabolic process (GO:0006805) involved in stress response, complement activation (GO:0006956) involved in immune system, lipid metabolic process (GO:0006629) and cellular response to estrogen stimulus (GO:0071391) (Figure 4).

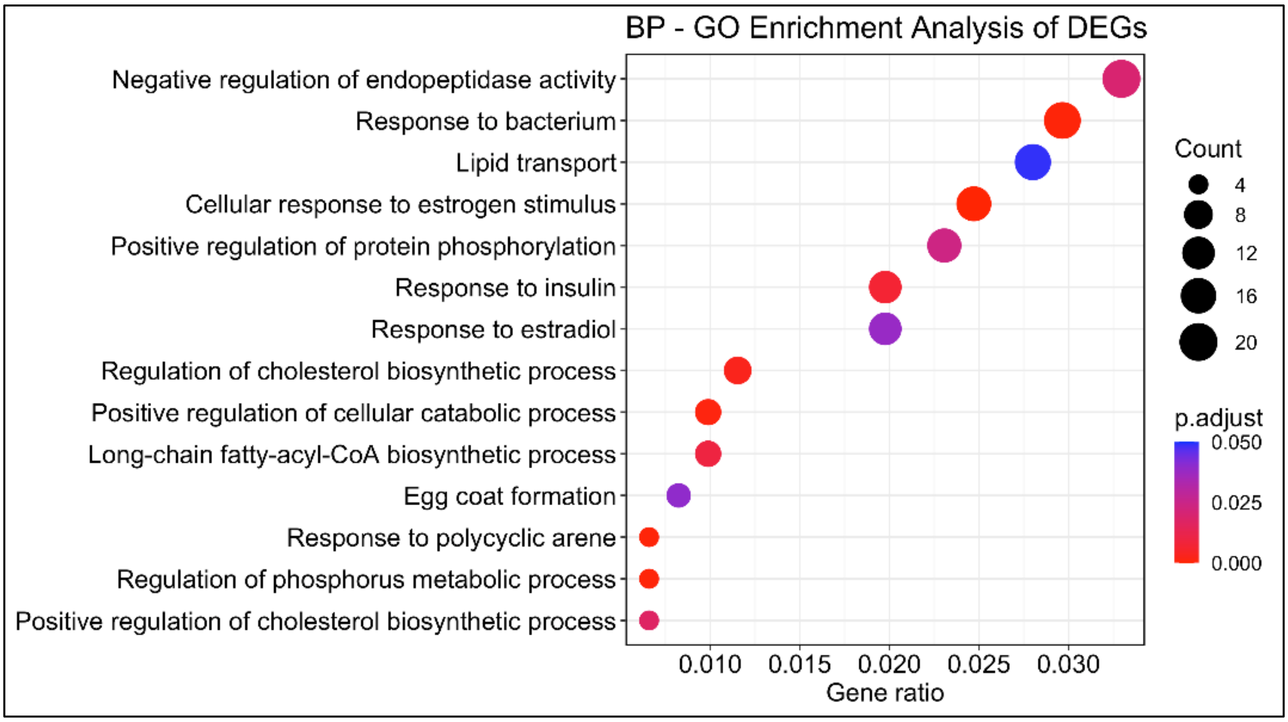

Figure 3 - Gene ontology enrichment analysis of differentially expressed genes (DEGs) between livers of immature (*n* = 3) and mature (*n* = 3) females. *y*-axis indicates the GO term description; *x*-axis indicates the gene ratio. The size of the dot is based on gene count, and the color of the dot shows the GO term's enrichment significance (*p*-adjust < 0.05).

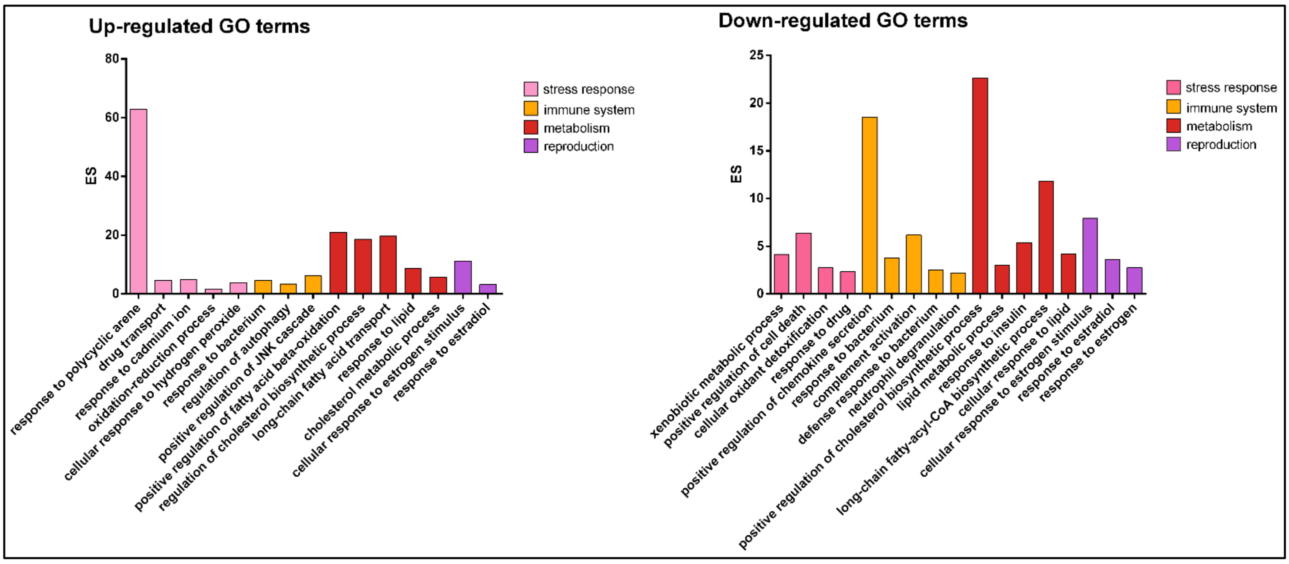

Figure 4 - The up- and downregulated GO terms examined based on their role in immune system, metabolism, reproduction and stress response. *y*-axis indicates the enrichment score (ES); *x*-axis indicates the GO term description. The colors indicate the processes examined.

KEGG Enrichment and BRITE Analysis

In order to investigate the dynamics occurring in the swordfish liver, 203 upregulated and 105 downregulated genes with KEGG annotation were mapped onto the Kyoto Encyclopedia of Genes and Genomes database. KEGG BRITE functional hierarchies analysis was performed at A (macrocategories) and B (subcategories) levels using the number of DEGs as the variable (Figure 5 and Figure 6). The most representative KEGG macrocategories were related to metabolism (DEGs > 100), response to environmental information processing (DEGs > 60), cellular processes (DEGs > 15) and human diseases (DEGs > 10). The most representative subcategories were related to lipid metabolism (DEGs > 30), transport and catabolism (DEGs > 10) and immune system (DEGs > 5). The KEGG enrichment analysis revealed a total of 15 enriched pathways, 6 upregulated and 9 downregulated. Among them, in mature females, steroid biosynthesis (*map00100*) and fatty acids degradation were upregulated, while metabolism of xenobiotics by cytochrome P450 (*map00980*), complement and coagulation cascades (*map04610*) biosynthesis of unsaturated fatty acids were downregulated (Figure 7 and Figure 8).

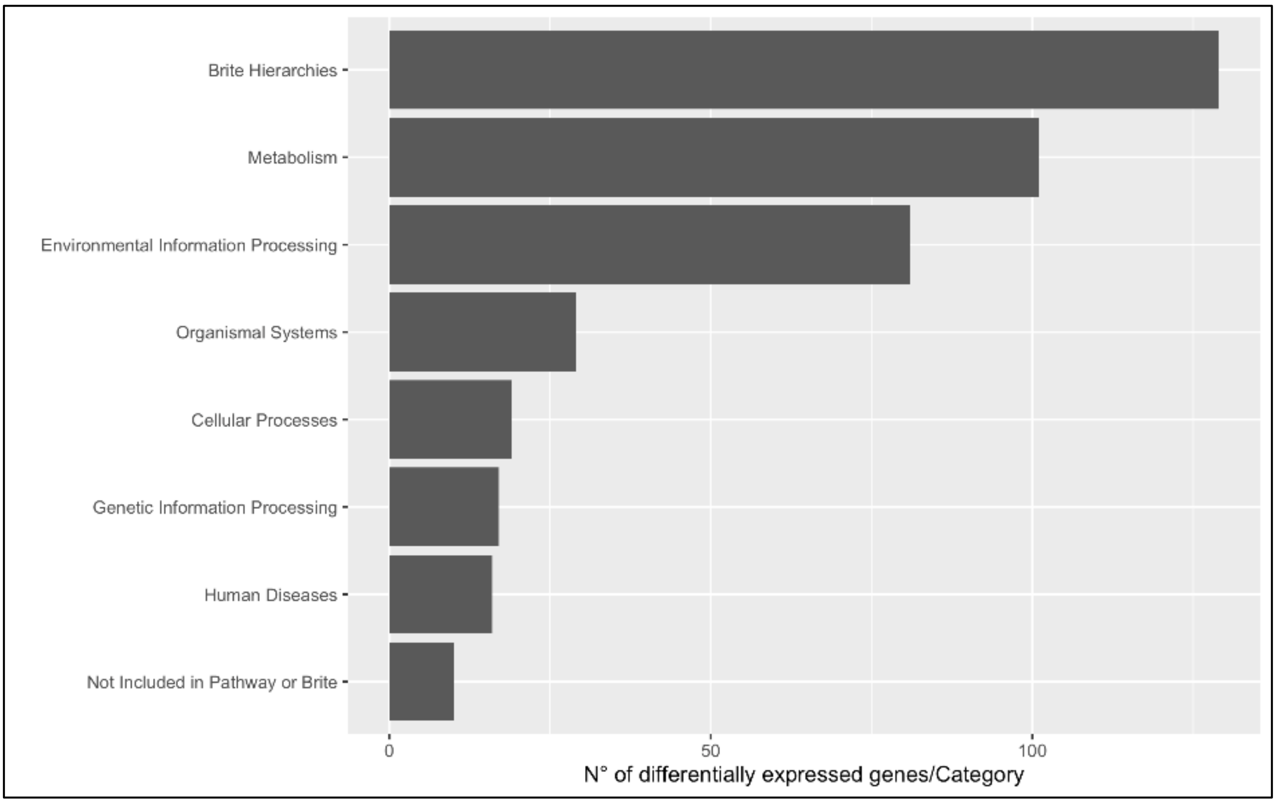

Figure 5 - KEGG BRITE functional hierarchies analysis category A (macro category). The bar plot shows in *x*-axis the number of DEGs per macrocategory between livers of immature (*n* = 3) and mature (*n* = 3) females; *y*-axis indicates the pathway description.

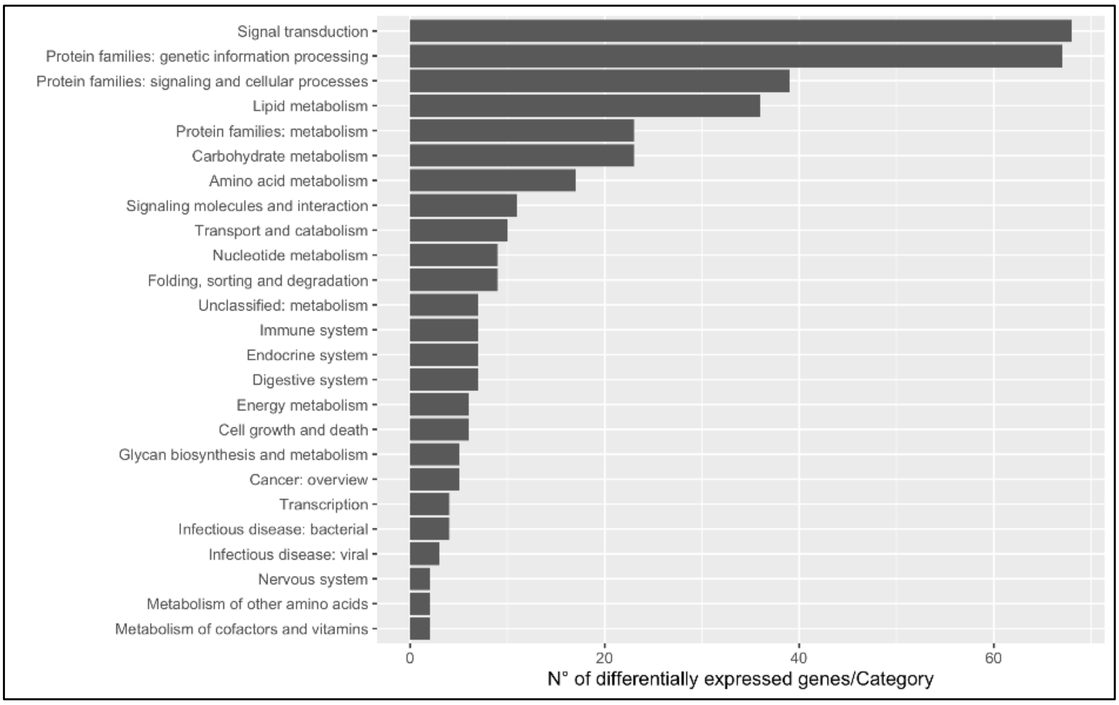

Figure 6 - KEGG BRITE functional hierarchies analysis category B (intermediate category). The bar plot shows on *x*-axis the number of DEGs per intermediate category between livers of immature (*n* = 3) and mature (*n* = 3) females; *y*-axis indicates the pathway description.

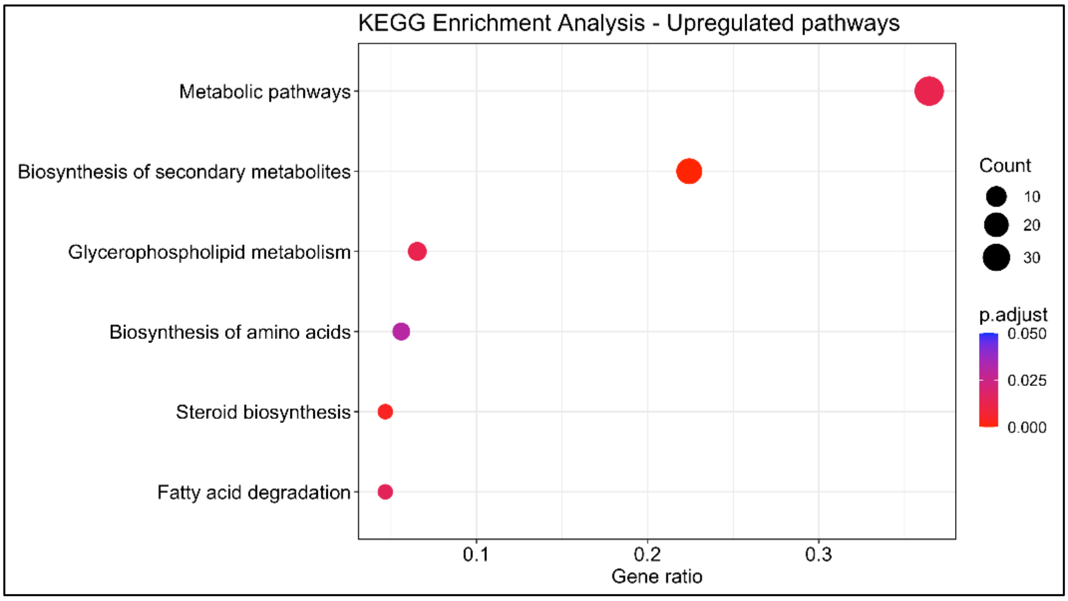

Figure 7 - KEGG enrichment analysis of differentially upregulated genes between livers of immature (*n* = 3) and mature (*n* = 3) females. *y*-axis indicates the pathway description; *x*-axis indicates the gene ratio. The size of the dot is based on gene count, and the colour of the dot shows the GO term's enrichment significance (*p*-adjust < 0.05).

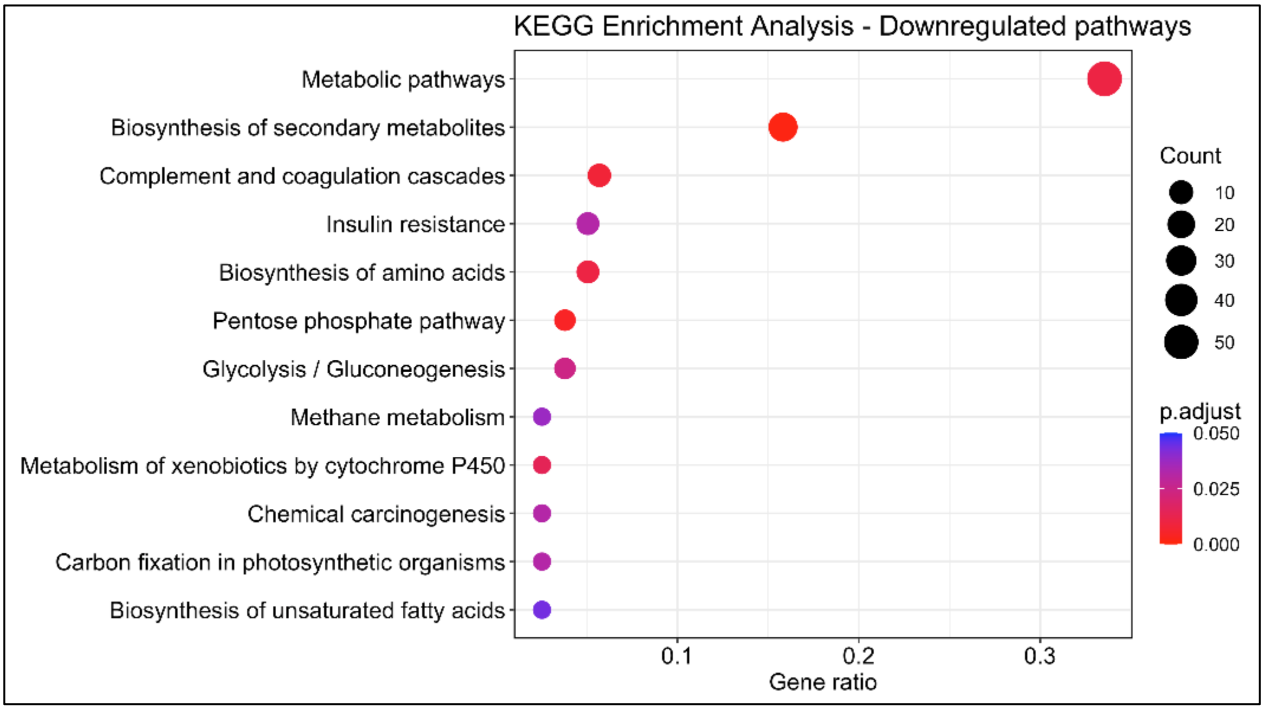

Figure 8 - KEGG enrichment analysis of differentially downregulated genes between livers of immature (*n* = 3) and mature (*n* = 3) females. *y*-axis indicates the pathway description; *x*-axis indicates the gene ratio. The size of the dot is based on gene count, and the colour of the dot shows the GO term's enrichment significance (*p*-adjust < 0.05).

Histological Analysis

Thanks to a histological classification according to Marisaldi et al., (2020) (Marisaldi et al., 2020), 22 immature and 28 mature females were identified. Among mature, 17 mature females showed a developing ovary, while 11 mature females showed a spawning capable ovary.

The histological appearance of female livers is shown in Figure 9. Liver parenchyma appeared homogeneous with polygonal-shaped hepatocytes having spherical nuclei; the lipids appeared as white dots and were distributed homogenously. MMs were located in all parenchyma and near blood vessels and sinusoids and appeared as dark brown dots. MMCs were located attached to the blood vessels or bile ducts and appeared with a colour ranging from dark brown to light brown; structured and unstructured morphologies and highly varied sizes were evident.

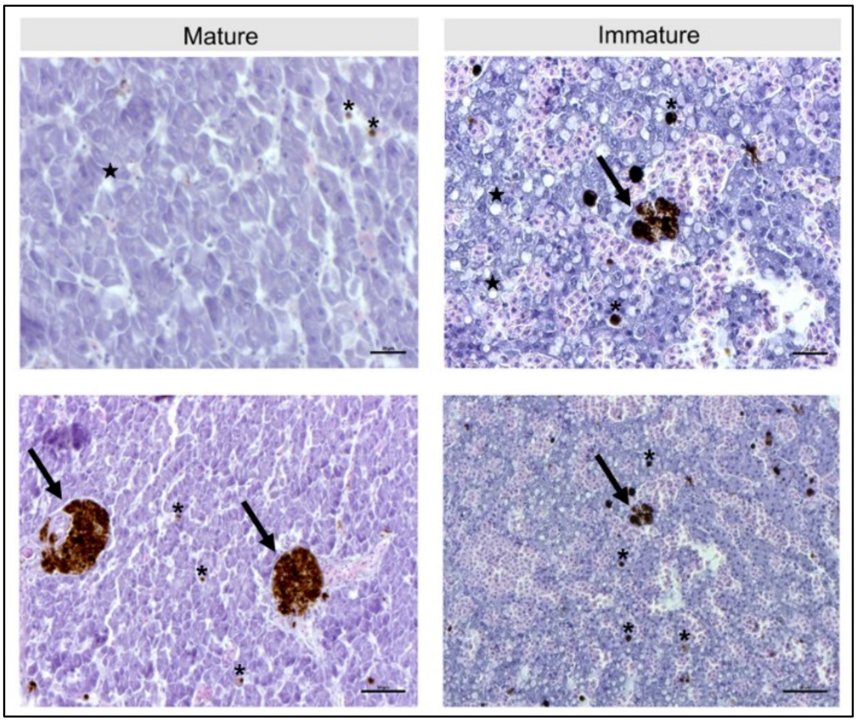

Figure 9 - Photomicrograph of the immature and mature liver of *Xiphias gladius*. Hematoxylin and eosin (H&E) staining of a section of the liver shows lipid components (red arrow); melanomacrophage center presence (yellow circle); single melanomacrophages (red circles).

MMs and MMCs related to fish size.

The density and number of MMCs and MMs were correlated with fish size (LJFL and TW) (Table 1). A significant positive correlation was observed between fish weight and both density and number of MMCs (*p*-value < 0.05, Pearson's correlation = 0.64 and 0.81, dMMCs and MMCs/mm^2, respectively). A similar result was observed between fish length and both density and number of MMCs (*p*-value < 0.05, Pearson’s correlation = 0.62 and 0.83, dMMCs and MMCs/mm^2, respectively). In contrast, a significant negative correlation was observed between fish weight and dMMs (*p*-value < 0.05, Pearson’s correlation = −0.53), whereas no significant correlation was observed between fish weight and MMs/mm^2 (*p*-value > 0.05, Pearson’s correlation = −0.43). A significant negative correlation was observed between fish length and both density and number of MMs (*p*-value < 0.05, Pearson’s correlation = −0.6283032 and −0.5467768, dMMs and MMs/mm^2, respectively).

	Length	**Weight**	**dMMC**	**dMM**	**MMC/mm^2**	**MM/mm^2**
Length	1	**0.982066**	**0.627270**	**−0.628303**	**0.820992**	**−0.546776**
Weight	**0.982066**	1	**0.642745**	**−0.532282**	**0.817944**	−0.436625
dMMC	**0.627270**	**0.642745**	1	−0.276178	**0.550470**	−0.244675
dMM	**−0.628303**	**−0.532282**	−0.276178	1	−0.442146	**0.954888**
MMC/mm^2	**0.820992**	**0.817944**	**0.550470**	−0.442146	1	−0.347469
MM/mm^2	**−0.546776**	−0.436625	−0.244675	**0.954888**	−0.347469	1

Significant correlations (*p* < 0.05) are displayed in bold

Table 1 - Pearson correlations between MMCs and MMs density (µm^2/mm^2) and number per mm^2 and fish biological parameters (LJFL and TW) (*n* = 50). Significant correlations (*p*-value < 0.05) are displayed in bold.

MMs and MMCs related to fish sexual maturity.

Differences in MMCs and MMs density and number were also investigated in relation to sexual maturity as histologically established.

The density of MMCs showed a significant increase in mature females compared to immature females (*p*-value < 0.05), while the density of MMs showed a strong significant decrease in mature females with respect to immature females (*p*-value < 0.001). The number of MMCs per mm^2 liver parenchyma showed a strong significant increase in mature females compared to immature females (*p*-value < 0.001), while the number of MMs per mm^2 showed a strong decrease in mature females compared to immature females (*p*-value < 0.001) (Figure 10).

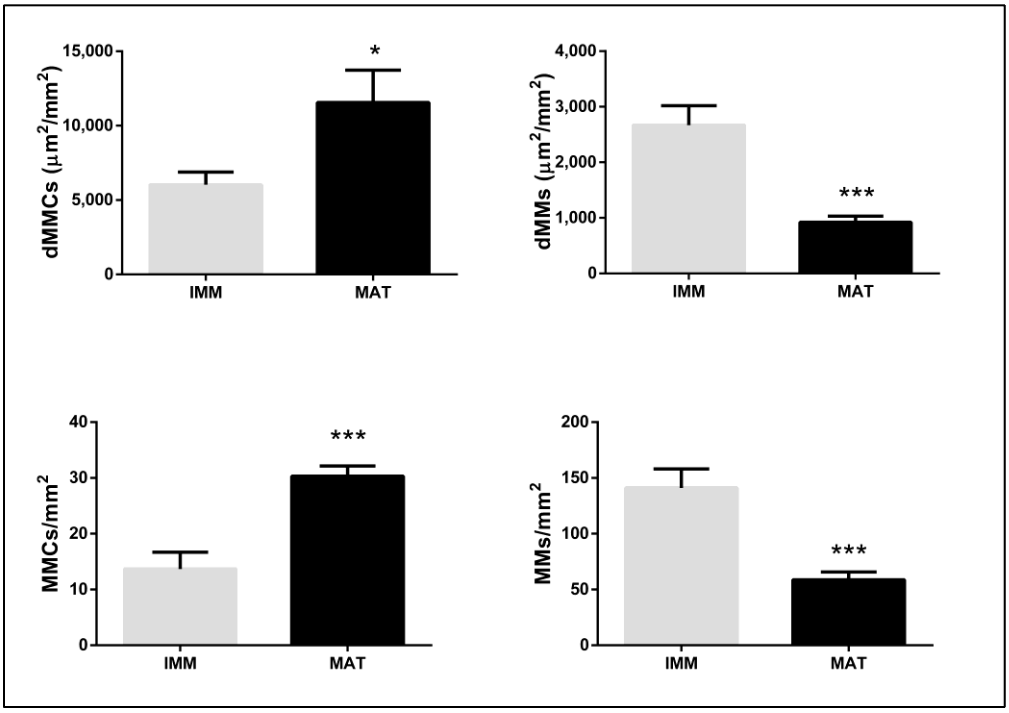

Figure 10 - Differences in density and number per mm^2 of single (MMs) melanomacrophages and centers (MMCs) between livers of immature (IMM; *n* = 22) and mature (MAT; *n* = 28) females. Asterisk (*) indicates significant statistical difference (* = *p*-value < 0.05, *** = *p*-value < 0.001).

Lipids related to fish size and sexual maturity.

The density of lipids was correlated to fish size (LJFL and TW) (Table 2).

A negative but not statistically significant correlation was observed between fish weight and lipids (p-value > 0.05, Pearson's correlation = −0.40). Instead, a significant negative correlation was observed between fish length and lipids (p-value > 0.05, Pearson's correlation = −0.53) (Table 2). Moreover, the density of lipids was related to sexual maturity. The density of lipids showed a significant decrease in mature females compared to immature females (p-value < 0.05) (Figure 11).

	Length	Weight	dLipids
Length	1	**0.982066**	**−0.52939**
Weight	**0.982066**	1	−0.40834
dLipids	**−0.52939**	−0.40834	1

Significant correlations (p < 0.05) are displayed in bold

Table 2 - Pearson correlations between lipid density ($\mu m^2/mm^2$) and fish biological parameters (LJFL and TW) (*N* = 50). Significant correlations (*p*-value < 0.05) are displayed in bold.

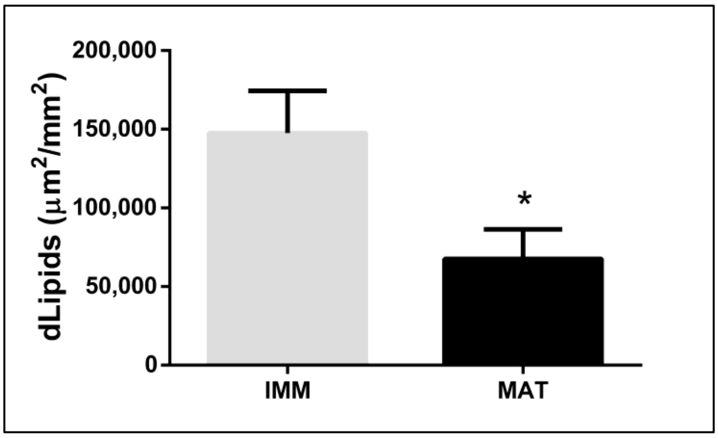

Figure 11 - Difference in lipid density between livers of immature (*n* = 22) and mature (*n* = 28) females. Asterisk (*) indicates a significant statistical difference for *p*-value < 0.05.

1.5 Discussion

The high-throughput sequencing technology has become a good method to identify and characterize the crosstalk and interactions among many biological processes (Hong et al., 2020). Furthermore, the coupling of RNA-seq with histological analysis can elucidate the dynamics occurring during the reproductive season and the sexual maturation of marine fish species and support a science-based decision-making pro-cess in the context of fishery management (May et al., 2019; Limonta et al., 2019; Hook et al., 2018). In the present study, we highlighted the differences in metabolism, reproduction, immune system, and stress response between immature and mature swordfish females during the breeding season. Focusing on metabolism, a higher density of lipids was found in the liver of immature females than mature females. A similar result was described by Zudaire et al., (2014) (Zudaire et al., 2014) in yellowfin tuna (*Thunnus albacares*). These results could be attributed to a different use of energy between mature and immature females. In mature females during the reproductive season, lipids were used for the hepatic synthesis of vitellogenin and neutral lipids which will be uptaken by the oocytes during vitellogenesis (Wiegand, 1996; Zudaire et al., 2014). These results were also confirmed by transcriptomic analysis. The GOEA evidenced a prevalence of up-regulated GO terms related to lipid transport and mobilization in mature females. On the contrary, the GO terms involved in the biosynthesis of metabolites, including fatty acids, are downregulated and this result could explain the lower lipid density found in the liver of mature females. In immature females, the lipids were used for the somatic growth, as confirmed by the overexpression of genes such as *igf1*, *igfbp2*, *ghe*, *Irs1*. The mature females, investing energies in reproduction, couldn't have enough energy to invest in immune system and to withstand the stress. Effectively, the mature females showed a downregulation of genes related to immune system (*il4r, c8b, c9*) and detoxification (*cyp1a1, cyp1b1*), while an up-regulation of genes related to the response to polycyclic aromatic hydrocarbons (*arntl2*) and response to cadmium (*hspa1s*). In addition, Casanova-Nakayama et al.'s (Casanova-Nakayama et al., 2011) study of rainbow trout (*Oncorhynchus mykiss*) indicated that the estrogens, present at high levels in mature females during the reproductive period (Seemann et al., 2015), could inhibit the immune system, inducing immunosuppressive effects and therefore expose the animal to infections. In addition, several studies showed an inhibitory action of the estrogens towards interleukins (*il*) (Burgos-Aceves et al., 2016; Shivers et al., 2015). *Xiphias gladius* is an apical predator and is considered a "reservoir" of pollutants. The presence of high concentrations of persistent organic pollutants (Pops) and trace metals (cadmium, mercury, arsenic) was previously confirmed in the liver of Mediterranean swordfish by other studies (Corsolini et al., 2008; Storelli & Marcotrigiano, 2006). Many of these compounds interact with the aryl hydrocarbon receptor (*ahr*), which binds a nuclear translocator (*arnt*). This complex binding specific DNA sequences, the xenobiotic response elements (*xre*), activate the transcription of genes encoding for enzymes (*cyp1a1*, *cyp1a2*, *cyp1b1*) involved in the detoxification of xenobiotics and drugs (Mortensen & Arukwe, 2007). Our results showed a downregulation of *cyp1a1* and *cyp1b1*, while *arnt2* was upregulated. These results could be explained by the fact that in the presence of high levels of estradiol, *arnt2* bind estrogen receptors (ERs) inducing several mechanisms including, both up and down regulation of ERs transcription and degradation of ERs (proteosome) (Gräns et al., 2010; Matthews & Gustafsson, 2006; Rüegg et al., 2008; Swedenborg & Pongratz, 2010). In this light, the competition between AhR and ERs for the same co-factor (arnt2) could inhibit cytochrome p450 signaling pathway (Safe & Wormke, 2003) and modulate the ERs gene expression, suggesting that the detoxification capacity of *cyps* is reduced in mature females characterized by high levels of estrogen due to the reproductive regulation. The transcriptomic results are in agreement with the histological ones. MMCs are involved in destruction/recycle of various exogenous and endogenous materials (Agius & Roberts, 2003; Passantino et al., 2014; Steinel & Bolnick, 2017; Widdicombe et al., 2020). In our study, the mature females showed a significantly higher density of MMCs than immature females. This result suggests that the increase of MMCs could be due to the downregulation of genes involved in the xenobiotics metabolism, such as *cyp1a1* and *cyp1b1*, and therefore, melanomacrophages are the only mechanism that undertake the detoxification function. In this study, the increase of density and number of MMCs were found to be positively and significantly correlated to the fish size (LJFL and TW). These results are in agreement with the positive correlation between MMCs and the age previously described in several teleost species (Carreras-Colom et al., 2022; Imai et al., 2022). Notably, a recent study on European anchovy demonstrates that long-term exposure to contaminated waters increases the presence and density of MMCs and MMs. Indeed, these results suggest that mature females, which are older than immature ones, are exposed for a long time to stressors or pollutants. Furthermore, the immature females showed a higher density of MMs compared to mature females. This result is in accordance with transcriptomic ones, which revealed that the immature females respond to pollutants by a more reactive immune and detoxification system. In addition, the immature females are not in reproduction, suggesting that the pathway involved detoxification is not inhibited by estrogens, as confirmed by the transcriptomic analysis. Moreover, the immature females aren't exposed to stressors or contamination for a long period: in fact, the density and the number of MMs is significantly correlated in a negative way to the fish size, suggesting that the immature females are exposed to stress for a while, then to acute stress. In *Poecilia reticulata* was evidenced that the number and density of MMs increased until 7-month and decreased after this age (Imai et al., 2022), while the density and number of MMCs increased afterwards 7-month. Our study indicates that immature and mature females show differences during the reproductive season, both in the number and density of melanomacrophages, in the hepatic lipid density, and in the expression of the genes. In addition, both swordfish females show a no optimal health status, but mature females seem to have more difficulty responding to chemical and chronic stress during the reproductive season.

1.6 Conclusions

In conclusion, the results obtained in this work reveal that during the reproductive season, mature females invest most of their energy in reproduction instead of detoxification and immune response. For this reason, during the reproductive season, mature females may be more susceptible to environmental stress and pollutants, also due to the inhibition of detoxification and the immune system. In this light, further studies on mature and immature females during the nonreproductive season could add new information on the health status of Mediterranean swordfish, to assess whether the immune-deficient situation of mature females persists or if it is more linked to the reproductive season.

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CHAPTER 2

Oogenesis impairment in Swordfish (*Xiphias gladius*) caught in central Adriatic Sea: differences between mature and immature females

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2.1 Abstract

The swordfish (*Xiphias gladius*) is a cosmopolitan, highly migratory teleost species, and an important fishing resource. Since the Mediterranean stock is considered to be overfishing and declining (ICCAT, 2017), the evaluation of the gonadal and health status of immature and mature females during the reproductive and non-reproductive seasons could be of great importance, as well as the size ranges caught in the Central Adriatic Sea. 77 swordfish females caught in the Central Adriatic Sea were collected in summer and autumn 2021. For each animal, gonadal and total weights were measured, and the liver and gonad samples were stored for histological analysis. This study showed that most size ranges are those at which the females are still immature, contributing to the collapse of the stock. In addition, the assessment of histopathological biomarkers of oogenesis impairment, such as pre-vitellogenic atresia, necrosis, vacuolization of pre-vitellogenic oocytes, and the presence of infiltrates, were found in both immature and mature females but in different percentages. Histological analysis at the hepatic level revealed a high occurrence of single melanomacrophages (MMs) and melanomacrophage centers (MMCs). The results highlighted that the density and number of MMCs changed in relation to the size and sexual maturity of the females. Furthermore, mature females show a reduced availability of lipids as an energy source in the liver. The present findings reveal that the health of the liver and ovary of Mediterranean swordfish caught in the Central Adriatic Sea is compromised, and impairment of oogenesis can occur before the acquisition of sexual maturity, compromising the correct formation and quality of gametes. Furthermore, mature females show a higher density and number of MMCs and MMs, suggesting that they are more affected by environmental stress than immature females.

2.2 Introduction

Xiphias gladius is considered highly overfished in Mediterranean Sea, and a stock recovery program has been adopted to rebuild stock biomass to sustainable levels by the end of the current decade (ICCAT 2020). In the Mediterranean Sea, the reproductive season of swordfish is from June to September, with a peak in July (Romeo et al., 2008). The known spawning areas are in Western Sicily and the Strait of Messina (Corriero et al., 2004), the Strait of Gibraltar and the Balearic Islands (Abid et al., 2019; Macías et al., 2005; Romeo et al., 2008), and the Levantine Sea (Tuğrul Zahit Alıçlı, 2012). During the last few decades, the Mediterranean Sea has been subjected to climate change and pollutant accumulation due to an increase in anthropogenic activities (Cinnirella et al., 2013). The effect of environmental stress and pollutants on fish was investigated in several studies using histopathological approaches (Abdel-Kader & Mourad, 2019; Elgaml et al., 2019; Hou et al., 2018; Kaptaner, 2015; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021; Senarat et al., 2017; Singh Brraich & Jangu, 2015; Song et al., 2021; Yadav Rajkiya Mahavidyalaya Unnao et al., 2018). However, only a few studies have investigated the effects of pollutants on the health and gonadal status of swordfish (Damiano et al. 2011; Desantis et al. 2005; Storelli and Marcotrigiano 2006). The histopathology of gonads has gained increasing interest as an endpoint biomarker to assess the reproductive capacity and health status of fish affected by a wide variety of environmental stressors, including pollution (Spanò et al., 2004; Tillitt et al., 2010). During the last few decades, many reviews have summarized that gonads are especially good targets of histopathology because of their high sensitivity to environmental stresses and, once impaired, could lead to a decline in the population (Spanò et al., 2004; Tillitt et al., 2010). Ovarian histopathological biomarkers, such as pre-vitellogenic atresia, necrosis, vacuolization of pre-vitellogenic oocytes, and the presence of infiltrates, have been related to the effects of several pollutants in previous studies (Bhaisare et al., 2022; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021). The liver is one of the organs most involved in the detoxification and activation of the immune response in fish exposed to environmental stressors (Gernhöfer et al., 2001). A biomarker of environmental stress exposure is the massive presence of melanomacrophages (MMs) in the liver. MMs are phagocytic cells involved in the detoxification, destruction, and recycling of various endogenous materials, such as erythrocytes, and exogenous materials, including nanoparticles, parasites, and xenobiotics (Dang et al., 2019; Fishelson, 2006; Haaparanta et al., 1996; Mela et al., 2007; Nowak, Dang, Webber, Neumann, Bridle, Bermudez, Evans, et al., 2021; Qualhato et al., 2018; Stosik et al., 2019; Vigliano et al., 2006; Widdicombe et al., 2020). MMs circulate freely in blood vessels, pass through the intact vessel wall (through margination and diapedesis mechanisms), and migrate in the parenchyma of the liver, spleen, and/or kidney (Agius, 1980; Passantino et al., 2014). In the parenchyma, MMs can be single or can form aggregates or centers (MMCs) (Agius, 1980; Imai et al., 2022; Saunders et al., 2010). Furthermore, MMCs contain three different pigments, hemosiderin, lipofuscin, and melanin (Agius &

Roberts, 2003). Hemosiderin is derived from the degradation of erythrocytes (Agius & Roberts, 2003), and lipofuscin is derived from beta-oxidation of fatty acids induced by pollutant exposure or aging (Imai et al., 2022). Melanin (Kalita et al., 2019) is directly synthesized by MMCs and is involved in the neutralization of the free radicals released during cell membrane rupture (Qualhato et al., 2018). The number, size, and distribution of MMs and MMCs vary according to age, nutritional level, and exposure to environmental stress conditions (Fishelson, 2006). In particular, an increase in MMs density in Thunnus thynnus, Engraulis encrasicolus, and Clarias gariepinus was observed after exposure to endocrine destroyers, toxic components, and xenobiotics (Agius & Roberts, 2003; Basilone et al., 2018; Passantino et al., 2014; Passantino et al., 2020; Sayed et al., 2019). In addition to detoxification, the biosynthesis and stocking of lipids occurs in fish liver (Tocher, 2003). In fish females During the reproductive period, neutral fatty acids and vitellogenin synthesized in the liver are mobilized towards the ovary to be incorporated into the oocytes and constitute the yolk components (Hiraoka et al., 2019).

The present study aimed to understand the health and reproductive status of Xiphias gladius females caught in the Central Adriatic Sea during the summer and autumn. With this goal, histopathological changes in the ovaries were identified, evaluated, and related to sexual maturity, season, and size. In addition, the density and number of MMs and MMCs in the liver are related to sexual maturity, month, and size. Using a histological approach, we highlight the compromised health status and reproductive impairment of Mediterranean swordfish in the Central Adriatic Sea.

2.3 Materials and Methods

Sampling activities

The gutted weight (GW) of landed specimens (N= 579), caught in summer and autumn 2021 in central Adriatic Sea by a long liner, was recorded (min=7.7 kg; max=46.4 kg). The ovary and liver were collected from 77 specimens with a range size of 9.1 kg to 34 kg. Samples were collected according to the guidelines of the International Commission for the Conservation of Atlantic Tuna (ICCAT). The procedures did not include animal experimentation, and ethical approval was not necessary according to Italian legislation (D.L. 4 of March 2014, n. 26, art. 2).

Histological analysis

Samples of ovaries and livers (~3 cm^3) were fixed in a formaldehyde-glutaraldehyde solution (NaH2PO4·H2O+NaOH+formaldehyde 36.5%+glutaraldehyde 25%+H2O) and stored at 4 °C until histological analysis. The ovary and liver samples were serially dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Sections (4 µm) were cut using a microtome (model RM2125 RTS; Leica Biosystems, Wetzlar, Germany), stained with Mayer's hematoxylin and eosin, and examined under a microscope (Axio Imager 2; Zeiss, Oberkochen, Germany).

Histopathological analysis of ovaries

The reproductive status of the females was determined according to previous studies (Marisaldi et al., 2020; Saber et al., 2020). The analysis of ovarian alterations was performed in five sections, with a separation between each section of 50 µm, taken with a 10x objective. Alterations were identified according to the methods described by Nibamureke and Wagenaar (2020) and Rodrigues et al. (2021). The alterations of ovaries were divided into alterations of oocytes, such as pre-vitellogenic atresia and vacuolization of oocytes, and alterations of interstitial tissue, such as necrosis, fibrosis, and infiltration of white blood cells. Fibrosis was determined macroscopically, whereas the other alterations were determined microscopically. Histological alterations were considered to be present in an animal only if present in 75% of the sections analyzed. Subsequently, the percentage of females affected by single alterations was evaluated in relation to their sexual maturity, season, and size. Females were considered healthy when all alterations considered were totally absent.

MMCs, MMs and lipids evaluation in the liver

Quantification of MMCs and MMs was performed in five sections taken with a 20x objective (digital field area = 149,838 µm2), while the quantification of lipids was performed in five sections with a 40x objective (digital field area= 39,433 µm2) (Imai et al., 2022). The separation between sections was

50 µm. The density of MMCs and MMs, their number per mm2 of hepatic parenchyma, and the density of lipids were measured using Fiji (Schindelin et al., 2012). The density of MMCs, MMs, and lipids was expressed as µm2/mm2.

Statistical analysis

Data for MMCs, MMs, and lipids were first checked for normality using the Shapiro test. Pearson's correlation test was performed for the density and number of MMCs and MMs, the density of lipids, and GW (kg) in the R-studio environment using *the cor.test* command. The density and number of MMCs and MMs were analyzed in relation to size and sexual maturity/season by ANOVA and in relation to season and sexual maturity by t-test using GraphPad Prism 6 version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The density of lipids was analyzed in relation to size and sexual maturity/season by ANOVA and in relation to season and sexual maturity by t-test using GraphPad Prism 6 version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.4 Results

Swordfish size-frequency distributions

The overall and per-season size distributions of landed swordfish are shown in Figures 1 and 2, respectively. Overall swordfish mean size was 15.93 kg (GW), ranging in size between 7.7 and 46.6 kg GW. The most represented size ranges were 10-15 kg and 15-20 kg (Fig.1).

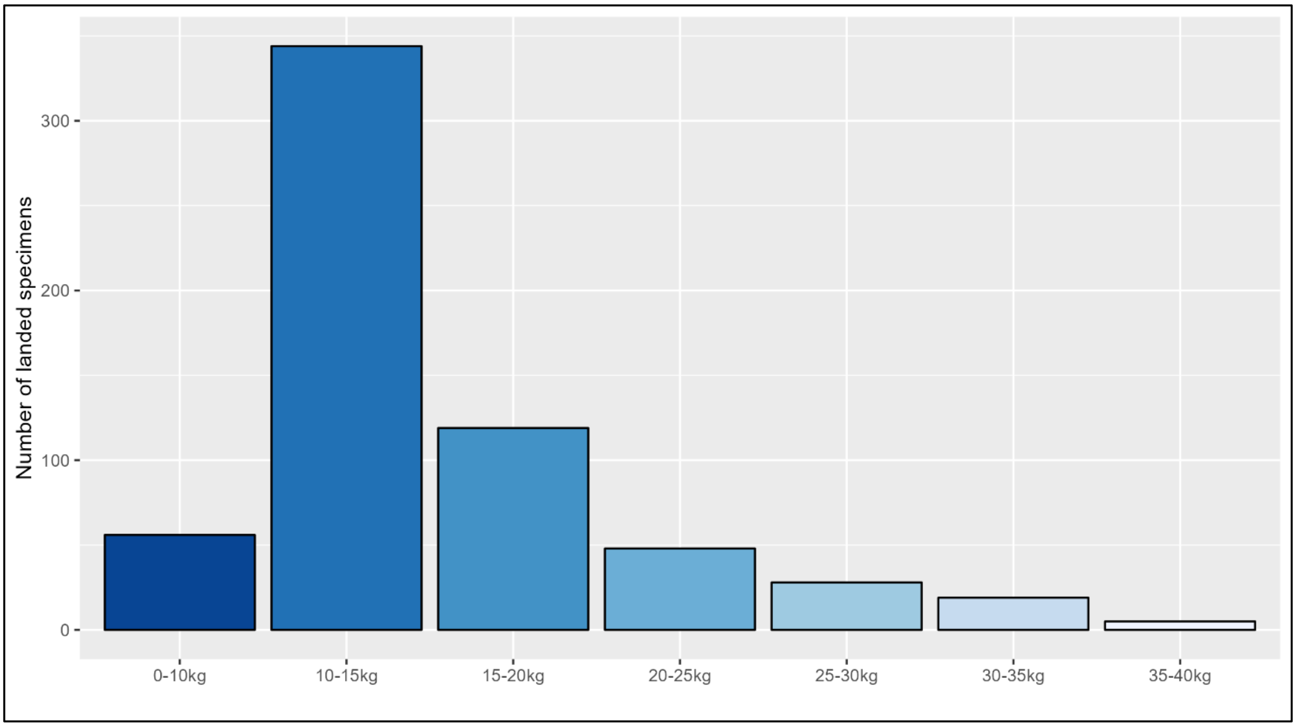

Figure 1 - Total size-frequency distribution. The color of the bar plot represents the size range.

In summer, the most represented size range was 10–15 kg, while in autumn, it was 10-15 kg and 15-20 kg (Fig. 2). Eighty% of the landed swordfish weighed under 20 kg.

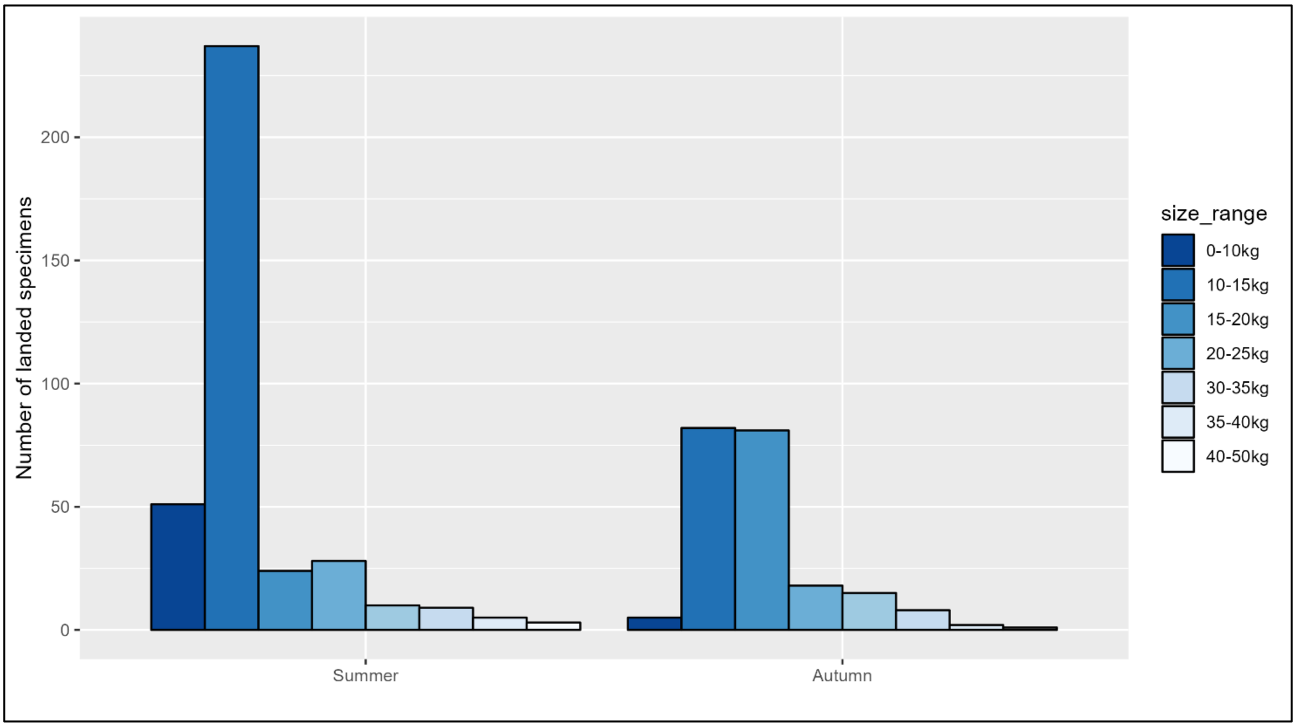

Figure 2 - Seasonal size-frequency distribution. The colour of bar plot indicates the size-range

Histopathological analysis of ovaries

According to Marisaldi et al., (2020) and Saber et al., 2020, 36 immature and 41 mature females were identified. The immature females showed compact ovigerous lamellae, with oogonia, chromatin nucleoli, and perinucleolar oocytes, and no evidence of oil droplets in oocytes. The mature females showed a regenerating ovary with oogonia and primary oocytes with circumnuclear oil droplets. Furthermore, five alterations were identified in the ovaries: two alterations of oocytes (atresia and vacuolation of pre-vitellogenic oocytes) and three interstitial tissues (necrosis, infiltrates of white blood cells, and fibrosis) (Fig. 3).

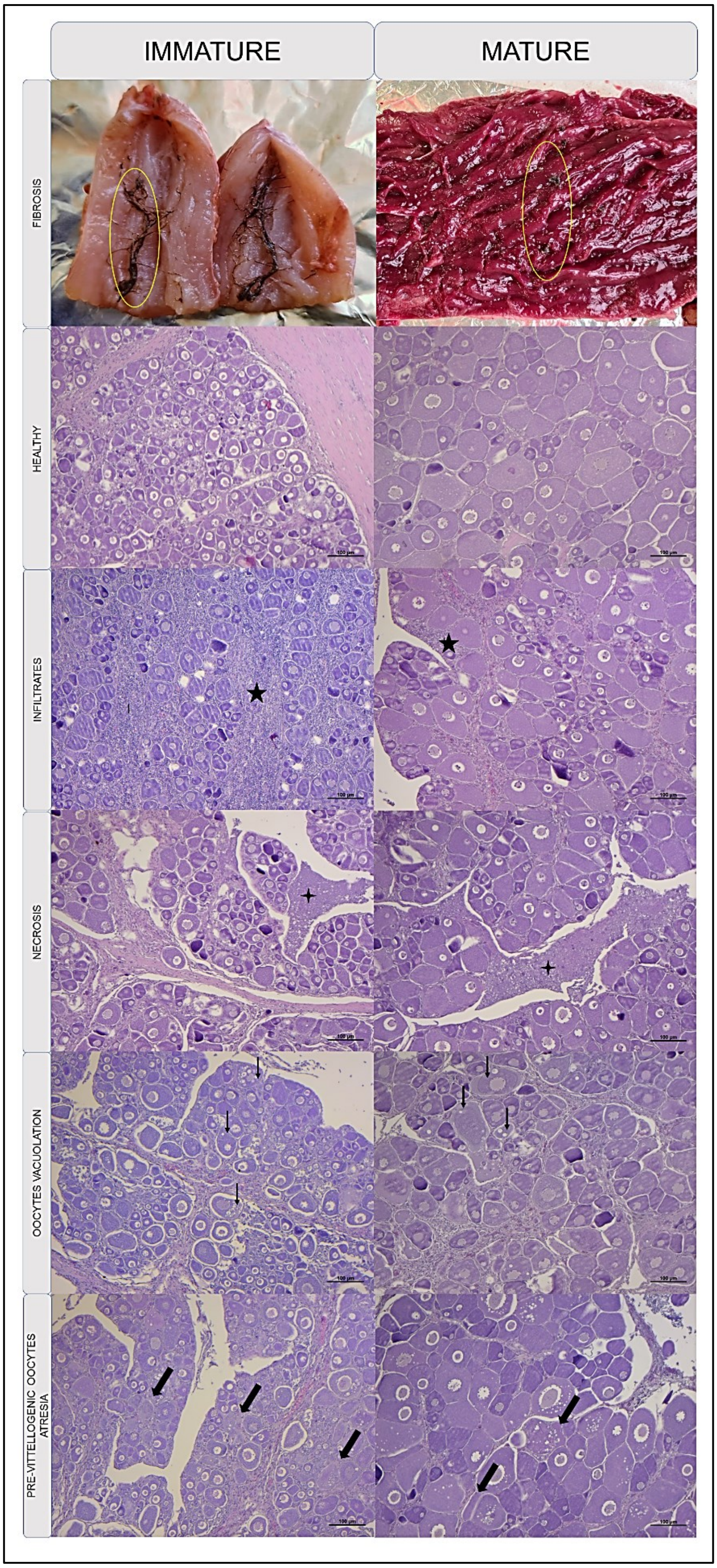

Figure 3 - Immature and mature ovaries: Fibrosis (Yellow circle); Healthy; Infiltrates of white blood cells (black star); Necrosis (black rhombus); pre-vitellogenic oocytes vacuolation (tiny arrow) and pre-vitellogenic atresia (bold arrow).

Only 4% of the females analyzed were healthy, while pre-vitellogenic atresia and vacuolation of oocytes were the most frequent alterations as well as fibrosis. (Fig. 4a). Investigating the occurrence of ovarian alterations in relation to sexual maturity, it was found that all healthy females (N=3) were mature. Fibrosis occurred most frequently in mature females, whereas infiltrates occurred most frequently in immature females. Vacuolation of oocytes and pre-vitellogenic atresia occurred in both the mature and immature females (Fig. 4b).

Investigating the occurrence of ovarian alterations in relation to the season, it was found that all healthy females were caught in autumn. Most vacuolated oocytes and pre-vitellogenic atresia were found in the summer. Most of the infiltrates were found in autumn (Fig. 4c).

Investigating the occurrence of ovarian alterations in relation to the size of females, it was found that all healthy females weighed found–the range 25-30 kg. Fibrosis was found in all size ranges, as well as oocyte vacuolation and previtellogenic atresia. Necrosis was found in the size ranges of 20–25 kg and 25–30 kg. White blood cell infiltrates were found in all size ranges except in the 20–25 kg range (Fig. 4d).

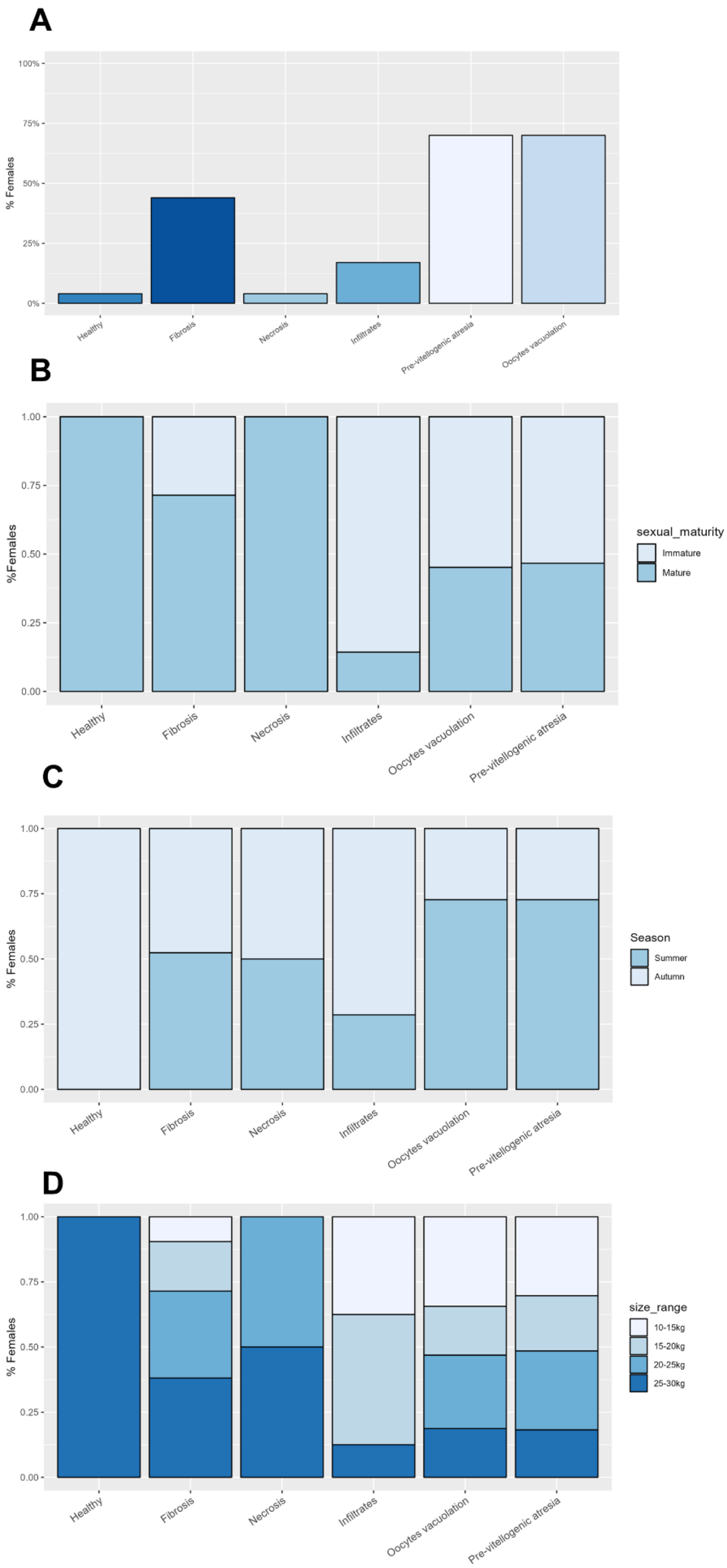

Figure 4 - Ovarian alteration frequencies: A) total; B) related to sexual maturity, C) related to season, and D) related to size range.

Histopathological analysis of livers

MMCs, MMs and Lipids quantification in relation to fish weight

The appearance of female livers is shown in Figure 5. The liver parenchyma appeared homogeneous with polygonal hepatocytes. The lipids appeared as white dots, and MMs were in all parenchyma and near blood vessels and were colored as dark brown dots. MMCs were attached to the blood vessels and appeared brown to light brown in color. Structured and unstructured morphologies and highly varied sizes are observed.

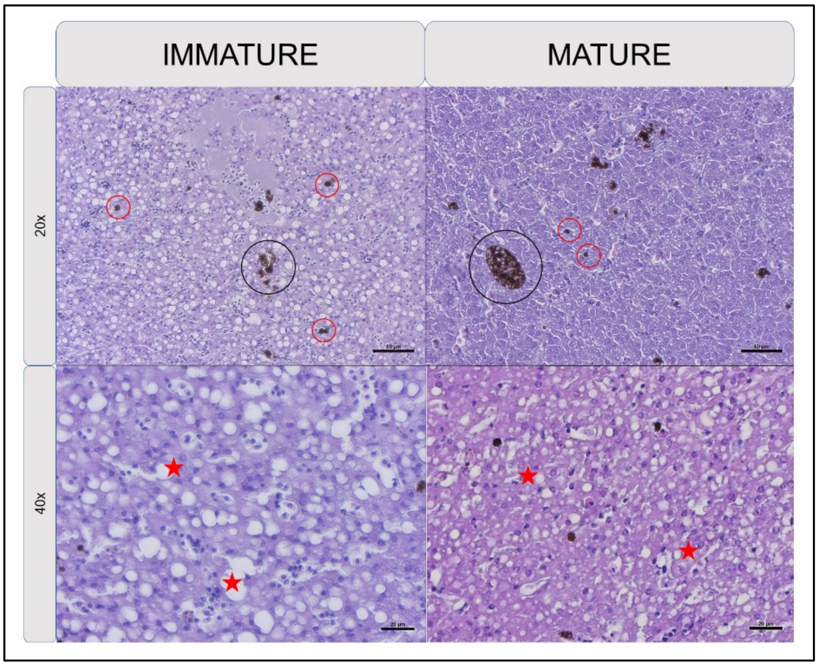

Figure 5 - Photomicrograph of the immature and mature liver: lipid (red star); melanomacrophage center (black circle); single melanomacrophages (red circle).

The density and number of MMCs and MMs were correlated to fish weight (GW) (Table 1). Between fish weight and both density and number of MMCs, a significant positive correlation was observed (p-value <0.05, Pearson’s correlation= 0.59 and 0.44, dMMCs and MMCs/mm^2 respectively). However, no significant correlation was observed between fish weight and either the density or number of MMs (p-value <0.05, Pearson’s correlation= 0.15 and 0.22).

	Weight	**dMMCs**	**dMMs**	**MMCs/mm^2**	**MMs/mm2**
Weight	**1**	**0.5959884**	0.1564423	**0.4454894**	0.2192134
dMMCs	**0.5959884**	**1**	**0.3012749**	**0.5762843**	0.2755039
dMMs	0.1564423	**0.3012749**	**1**	**0.4956766**	**0.7474606**
MMCs/mm^2	**0.4454894**	**0.5762843**	**0.4956766**	**1**	**0.5292198**
MMs/mm2	0.2192134	0.2755039	**0.7474606**	**0.5292198**	**1**

Table 1 - Pearson correlations between MMCs and MMs density (μm^2/mm^2), number per mm^2, and fish size (GW or kg). Significant correlations (p-value < 0.05) are indicated in bold.

A significant negative correlation was observed between fish weight and lipid content (p-value >0.05, Pearson's correlation= -0.30) (Table 2).

	Weight	**dLipids**
Weight	**1**	**-0.30312**
dLipids	**-0.30312**	**1**

Table 2 Pearson correlations between lipid density (μm^2/mm^2) and fish size (GW or kg). Significant correlations (p-value < 0.05) are indicated in bold.

MMCs and MMs quantification in relation to fish sexual maturity, size-range and season

MMCs and MMs were independently related to season, sexual maturity, and size range. No significant differences were found in the density or number of MMCs related to the season. A significant decrease in the density and number of MMs was observed between summer and autumn (p <0.05) (Fig. 6).

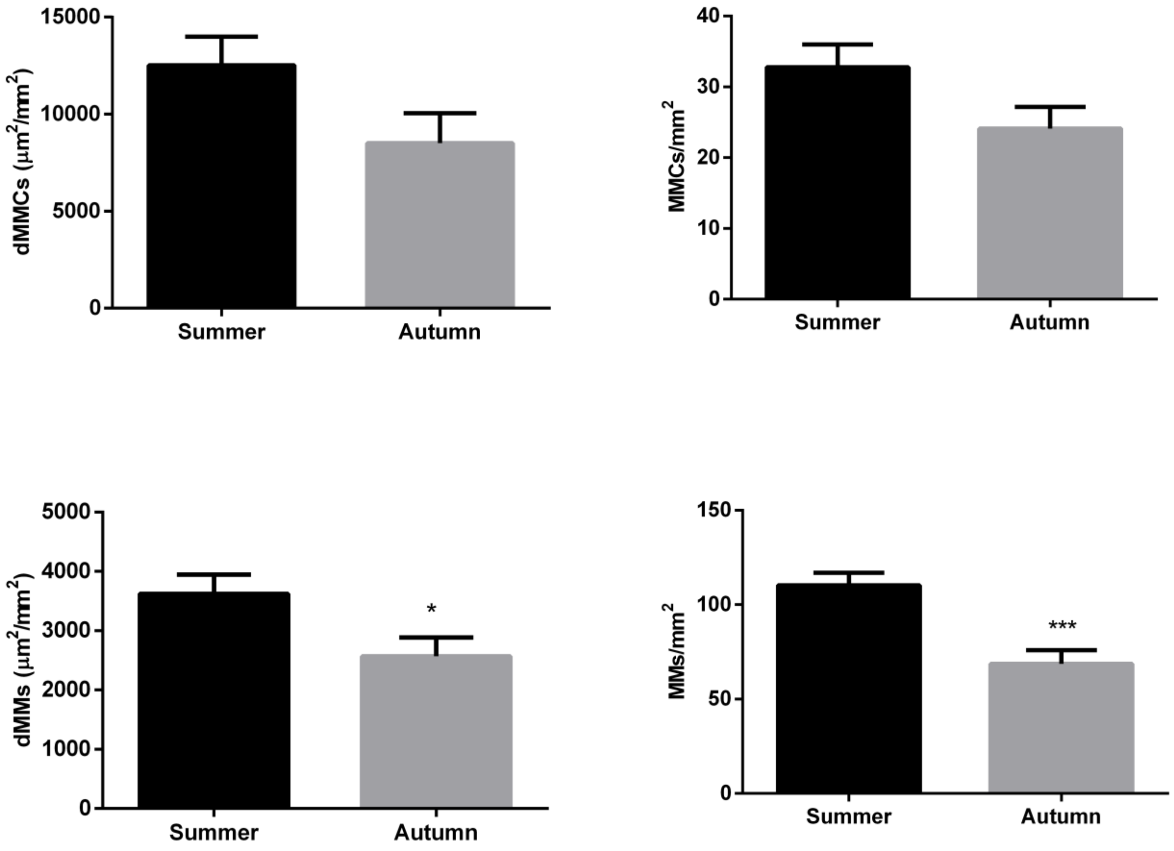

Figure 6 - Density and number per mm^2 of singles (MMs) melanomacrophages and centers (MMCs) related to season. * Indicates significant difference. (*= p-value <0.05, ***= p-value <0.001).

Furthermore, mature females showed a significantly higher density and number of MMCs and MMs than immature females (p-value <0.001) (Fig.7).

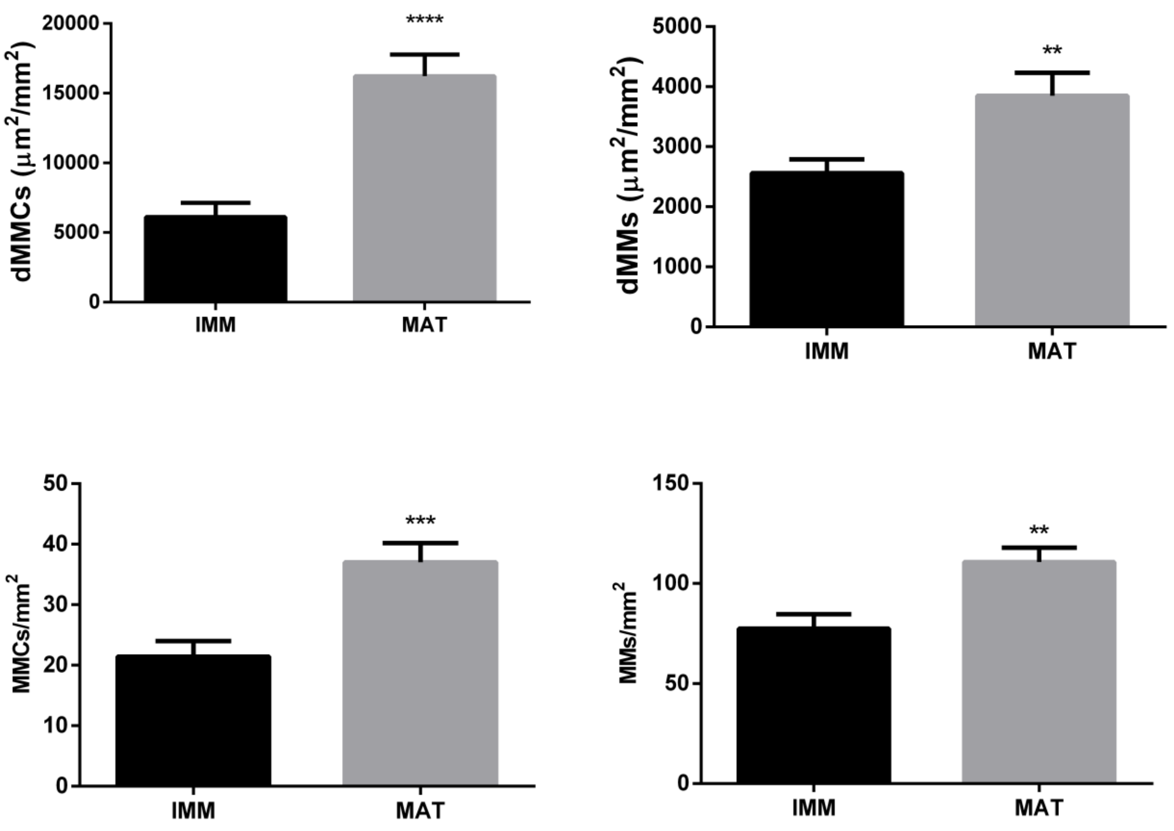

Figure 7 - Density and number per mm^2 of singles (MMs) melanomacrophages and centers (MMCs) related to sexual maturity. * Indicates significant difference. (**=p-value <0.01,***= p-value <0.001, ****= p-value <0.0001).

An increasing trend of density of MMCs and MMs and number of MMCs was found with increasing range size (p-value <0.05)(Fig.8).

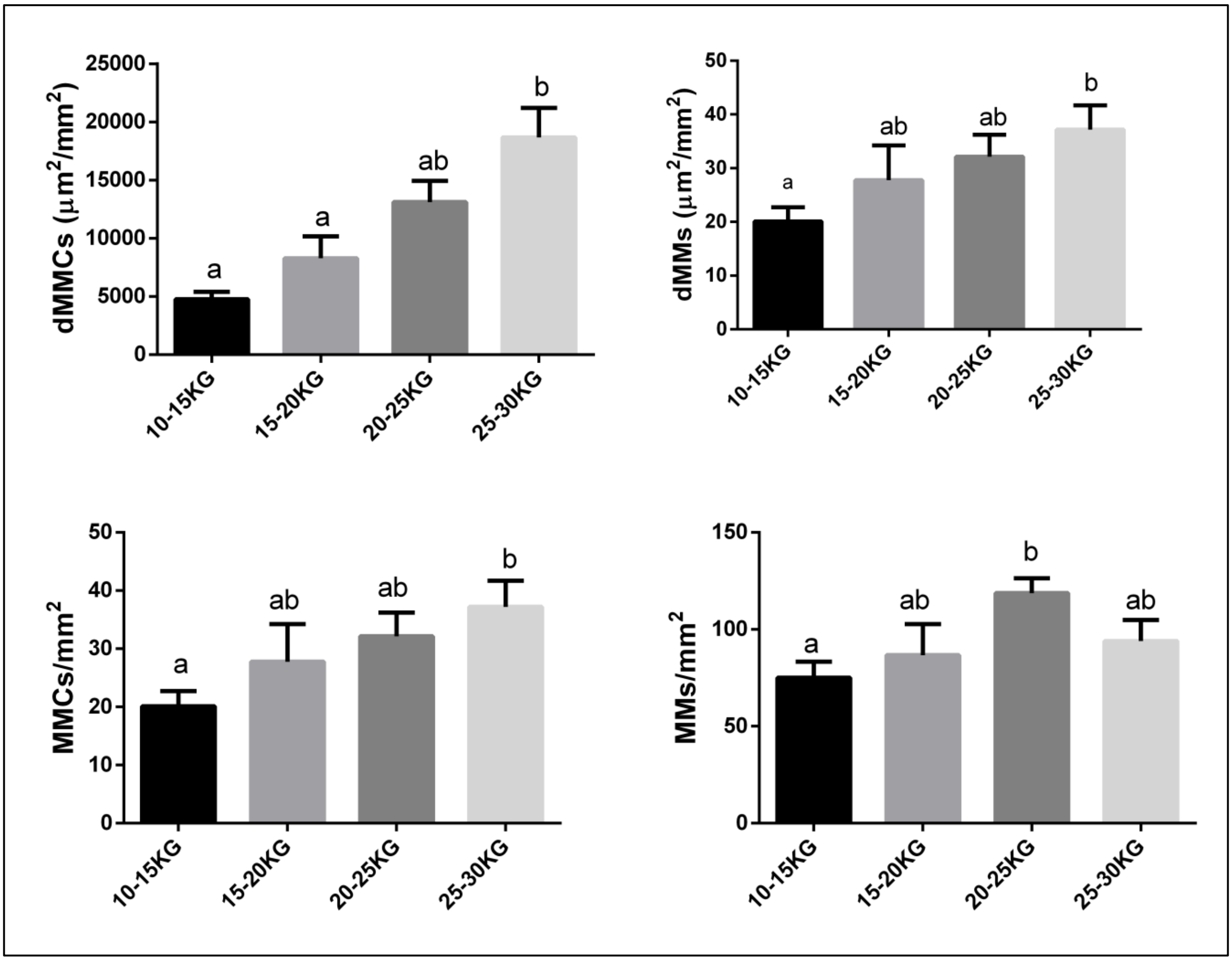

Figure 8 - Density and number per mm^2 of singles (MMs) melanomacrophages and centers (MMCs) related to size range. Different letters indicate significant differences.

MMCs and MMs density and the number were also investigated in relation to sexual maturity per season. The density of MMCs showed a significant increase in mature females caught in summer compared to immature females (p-value <0.05), whereas no significant differences were found between mature females and immature females caught in autumn. The density of MMs showed a significant increase in mature females caught in summer compared to immature females (p-value >0.05), while no significant differences were found between mature and immature females caught in autumn. The number of MMCs per mm^2 liver parenchyma showed a significant increase only in mature females caught in summer compared to immature females (p-value <0.05), and a similar result was found for the number of MMs per mm^2 ($p < 0.05$) (Fig. 9).

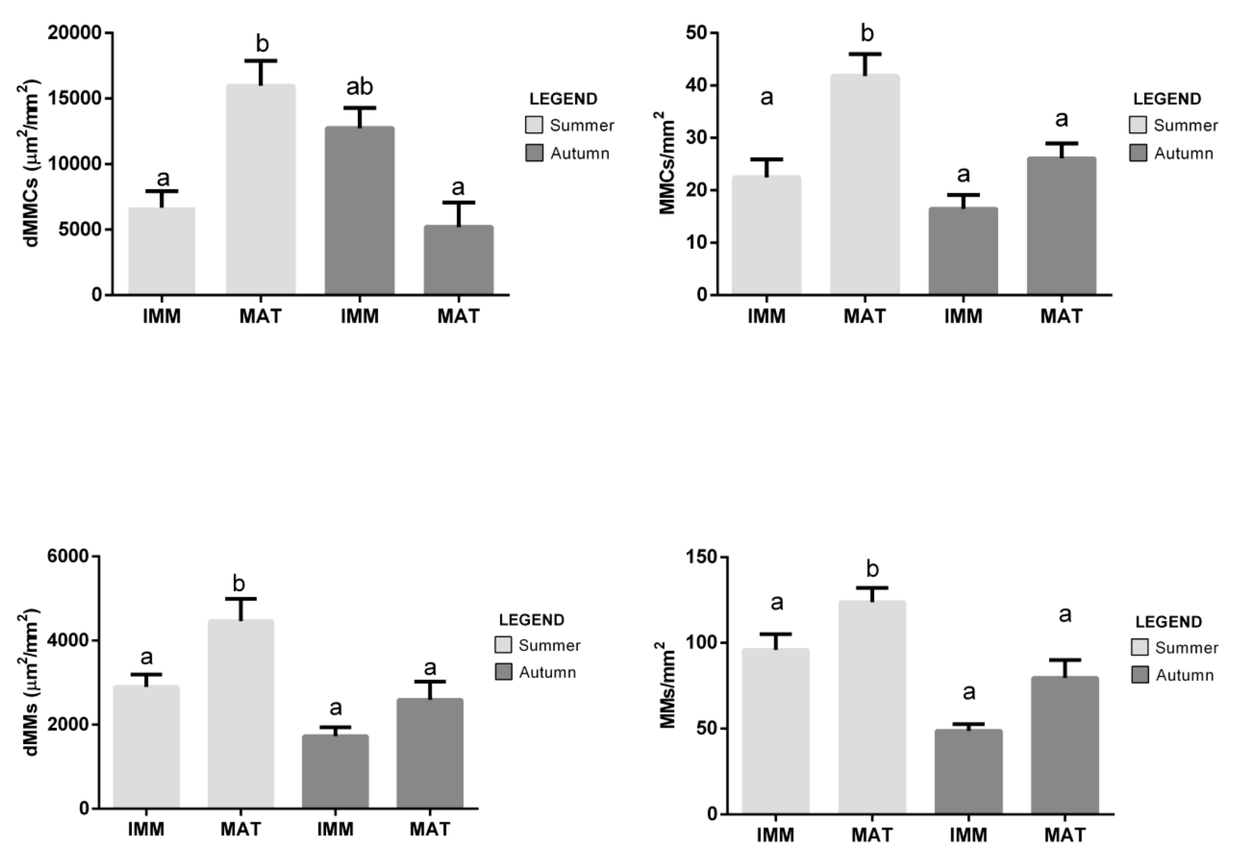

Figure 9 - Density and number per mm^2 of singles (MMs) melanomacrophages and centers (MMCs) related to sexual maturity/season. Different letters indicate significant differences.

Furthermore, the mature females caught in summer showed a higher density of MMCs than the mature females caught in autumn, and a similar result was found for the number of MMCs per mm^2. Mature females caught in summer showed a significant increase in density and number of MMs compared to mature females caught in autumn.

Lipids quantification in relation to fish sexual maturity, size and season

Lipid density was independently related to season, sexual maturity, and size range.

No significant differences were found in the density of lipids according to the season (Fig. 10). Furthermore, mature females showed a significant decrease in lipid density compared with immature females (p-value <0.001). The density of lipids decreased in the range of 20–25 kg compared to the other groups (p-value <0.05). Lipid density was also investigated in relation to sexual maturity per season. The density of lipids showed no significant differences between mature and immature females caught in summer and autumn (p-value <0.05), whereas mature females caught in summer showed a significant decrease compared to mature females caught in autumn (Fig. 11).

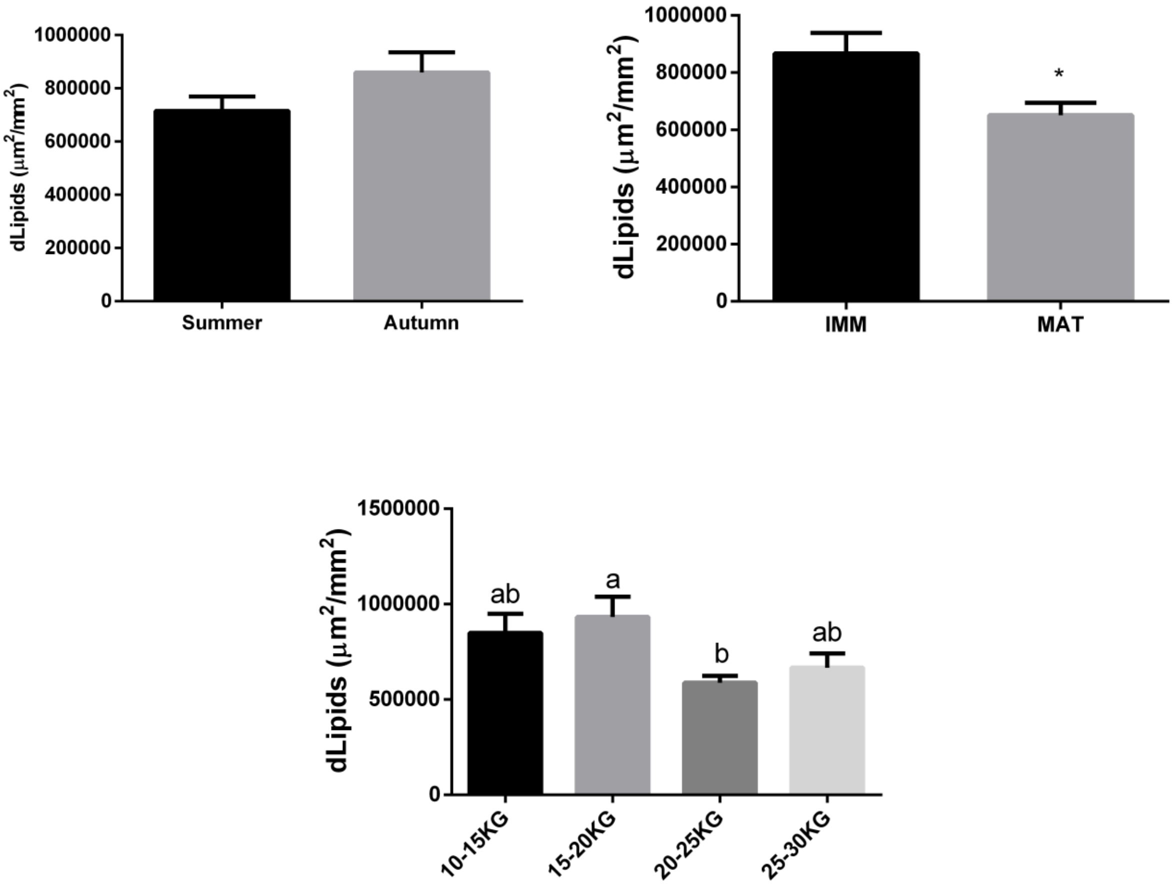

Figure 10 - Density of lipids related to sexual maturity, size range, and season. * Indicate significant difference (*= $p<0.05$) and different letters indicate significant differences.

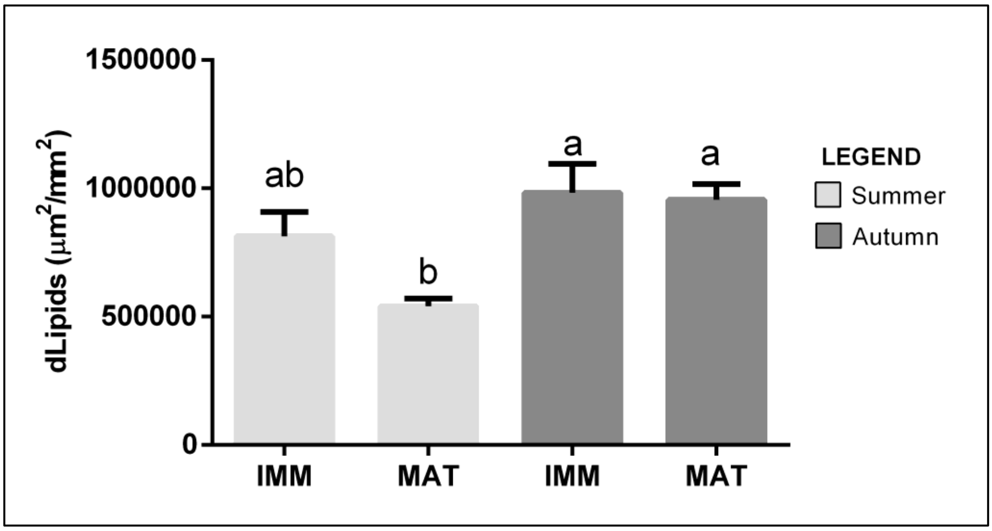

Figure 11 - Density in lipid density related to sexual maturity and season Different letters indicate significant differences.

2.5 Discussion

Xiphias gladius is an important fishing resource. In recent decades, the Mediterranean stock has been overfished, and the ICCAT has established a multi-annual recovery plan with annual monitoring of reproductive biology, abundance, and catch indices (ICCAT, 2019). The current ICCAT management measures for the conservation and restocking of the swordfish population establish a minimum catch size of 100 cm or 10 kg and fishing closure from January to March. This study demonstrated that the most landed size ranges are those at which the females are still immature, considering that they reach the L50 at 120-130 cm, unlike the males that reach sexual maturity at approximately 90 cm (ICCAT, 2019; De Metrio et al.,1989; De la Serna et al., 1996; Marisaldi et al., 2020). In addition, range sizes under 20 kg were caught in both seasons, with a peak in summer. It is likely that the juveniles (<20 kg) migrate to the Central Adriatic Sea for feeding before the older specimens, which are still engaged in reproduction. Additionally, five types of alterations were found in the ovaries, three in the interstitial tissue, and two in the oocytes. In our study, alterations in the ovary (vacuolation and atresia of previtellogenic oocytes, necrosis, fibrosis, and infiltration of white blood cells) were found in both immature and mature females. Only three mature females were healthy. A large proportion of mature females showed increased fibrosis and necrosis compared to immature females. In contrast, immature females showed more infiltrates of white blood cells than did mature females. In addition, in both summer and autumn, all females showed the same rate of alterations. Regarding alterations in oocytes, both immature and mature females showed similar results. Endocrine disruptors and other pollutants impair fish reproduction (Delbes et al., 2022; Abdel-Kader & Mourad, 2019; Elgaml et al., 2019; Hou et al., 2018; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021; Senarat et al., 2017; Singh Brraich & Jangu, 2015; Song et al., 2021; Yadav Rajkiya Mahavidyalaya Unnao et al., 2018). In particular, the impact of pollutant exposure in immature females results in damage to pre-vitellogenic oocytes and interstitial tissue, causing incorrect development of ovaries and a delay in acquiring sexual maturation. In addition, the exposure of mature females to pollutants induces infertility and arrests gonadal development (Delbes et al. 2022). Moreover, atresia, especially in previtellogenic oocytes, as found in the present study, is an important biomarker of pathological conditions due to exposure to environmental contaminants (Kaptaner and Ünal 2011). Puttipong et al., 2022 suggest that exposure to environmental contamination could cause alterations in the ovaries and testes of *Pangasianodon hypophthalmus*, and that atresia in previtellogenic oocytes may potentially result in decreased reproductive success of the fish. The compromised status of the ovaries was confirmed by quantification of MMCs and MMs in the liver. In our study, mature females showed a higher value of MMCs and MMs, density, and number of mm2 compared to immature females. Similar results have been previously reported for age, size, and exposure to chemical pollutants (Agius & Roberts, 2003; L. Passantino et al., 2014; Steinel & Bolnick, 2017; Widdicombe et al., 2020). In *Clarias gariepinus* and *Carassius auratus*, an increase in the density and number of MMCs in the liver was observed after exposure to high nonylphenol concentrations. In *Engraulis encrasicolus,* caught in contaminated areas, an increase in MMCs density was found compared to that in protected marine areas. Moreover, an increase in MMCs density from age 1 to age 2 of European anchovies was found in fish caught in all areas (contaminated or not), suggesting a role of MMCs in the aging processes (Basilone et al., 2018). In the present study, the highest values of density and number of MMCs and MMs were found in mature females during the summer. These results suggest that mature females are more susceptible to environmental stressors during the reproductive season than during the non-reproductive season. In fact, the reduced storage of lipids evidenced in mature females during the summer could reflect a reduced energy store, which could not be sufficient to respond to environmental stress.

2.6 Conclusions

In conclusion, our study highlights for the first time that the health and reproductive status of Mediterranean swordfish caught in the Central Adriatic Sea are compromised. At the gonadal level, oocyte alterations occur in both immature and mature females, whereas fibrosis and necrosis occur primarily in mature females. These results suggest that oogenesis impairment can occur in immature females, compromising or delaying the acquisition of sexual maturity. In addition, at the hepatic level, mature females show a higher density and number of MMCs and MMs compared to immature females, suggesting that they are more affected by environmental stress. Further studies on males during the reproductive and non-reproductive seasons could add new information on the health status of Mediterranean swordfish to assess whether the health and gonadal status identified in the females persists in the male specimens.

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CHAPTER 3

ddRAD genotyping reveals new insight into the genetic structure and diversity, fitness and evolutionary potential of Atlantic and Mediterranean swordfish stocks

(MANUSCRIPT IN PREPARATION)

3.1 Abstract

In fisheries management, use of advanced genetics tools has proven as an effective solution not only to define genetic structure and diversity of fisheries stocks but also stock status and its capacity to face changing environmental conditions. Indeed, it is important to apply conservation genetics to fisheries management to foster a science-based decision-making process that can be used to create practical management measures. However, in the case of highly migratory marine species such as the swordfish (Xiphias gladius) this strategy is rather challenging. For these reasons, the objectives of the present study were to 1) gain deeper insights into the genetic structure and genetic diversity at the genomic level of the three swordfish Atlantic stocks: the North Atlantic, the South Atlantic and the Mediterranean; 2) to assess the fitness and evolutionary potential of the three stocks; and 3) to confirm the current spatial stock boundaries and degree of mixture between the stocks. To pursue such objectives, we leveraged the power of de novo genome assembly and double digest restriction associated DNA (ddRAD) sequencing on 576 swordfish sampled from the three stocks. Multivariate and individual-based analysis of 26,324 SNPs showed substantial genetic differentiation between the Mediterranean and Atlantic stocks, while weak differentiation between the North and South Atlantic stocks was found. Furthermore, there were signs of slight genetic differentiation between east and west, both in the North and South Atlantic stocks. In addition, thanks to the allelic richness analysis at genomic level, it was possible to identify for the first time, genetic vulnerabilities of the Mediterranean stock which is losing allelic richness of important genes involved in detoxification, immune response, vitamin up-take and metabolism and serotonin signaling. The results of this study challenge current assumptions of the boundaries of the three stocks. Specifically, the South Atlantic population appears to occur beyond the currently used 5°N ICCAT management boundary, mixing with the North Atlantic population up to latitudes of 25°N. Likewise, the Mediterranean population can occur beyond the current management boundary at the Strait of Gibraltar, into a mixture zone in the Northeast Atlantic, where it mixes with the North Atlantic up to longitudes of 20°W. In this light, swordfish fisheries management should consider that in the East-North Atlantic area specimens belonging to all three stocks (10-14% of Mediterranean and 15% of South Atlantic) are caught and whose presence should be considered when genetic variability is monitored in this area. These results provide new and robust tools useful for a science-based decision-making strategy for the management of swordfish fisheries as well as a solid basis for an appropriate monitoring plan not only of the genetic structure and variability but also of the evolutionary potential of the three swordfish stocks.

3.2 Introduction

Effective management of wild populations relies upon knowledge of distribution, movements, habitat preferences, foraging habits and demographic trends, which, when integrated with genetics can refine the delineation of conservation units (Sveegaard et al., 2015; Yannic et al., 2016). Therefore, integrating genetics into the management of wild populations and understanding the processes shaping the genetic population structure represent two of the most compelling challenges in the field of conservation biology (Frankham, 2010a, 2010b; Holderegger et al., 2019; R. Taylor et al., 2017). The delineation of conservation units usually requires a two-step process: estimating the amount of gene flow between populations and evaluating the level of adaptive divergence (Allendorf et al., 2010; Palsbøll et al., 2006). The former can be assessed by genotyping loci to understand patterns of reproductive isolation, gene flow and demographic history of population, while the latter requires the study of functional genes and their distribution across the genome (Gagnaire et al., 2015). Such approaches are being revolutionized by the release of complete genomes at an unprecedented pace, which is transforming our understanding of levels and distribution of genetic variation and diversity in wild populations. Indeed, over the last two decades, conservation genetics has too benefited, as many other fields of study, from the advent of the next-generation sequencing, which enabled a genome-wide assessment of the genetic diversity and provided a more detailed scale of resolution and biological insights, such as evidence of deleterious mutations, signatures of selection, adaptive diversity and genetic erosion (Fuentes-Pardo & Ruzzante, 2017; Leroy et al., 2018). One of the most powerful applications of genomics technologies is the simultaneous estimation of parameters that require neutral loci (e.g., effective population size, migration rate, inbreeding) along with the identification of specific genomic regions under selection that can reflect adaptation to local conditions (Doyle et al., 2016; Vaux et al., 2021). In fisheries management, using genetics either alone or in combination with other approaches (e.g., otolith microchemistry, tagging, morphometrics) has proven as an effective solution to define connectivity and structure of fisheries stocks as well as to match spatial management units with the actual population structure (Cadrin et al., 2014; Cuéllar-Pinzón et al., 2016). Indeed, since the delineation of biological conservation units is essential for tailoring management to a species' range, environmental conditions, and local pressures (Reiss et al., 2009), it is important to apply conservation genetics to fisheries management to foster a science-based decision-making process that can be applied to design practical management measures. This genomic-based strategy becomes fundamental when assessing genetic population structure of highly migratory marine species such as tunas (*Thunnus* genera) and the swordfish (*Xiphias gladius*). Swordfish is a large epipelagic cosmopolitan species capable of long-distance movements as illustrated by both conventional (J. D. Neilson, 2007) and electronic (Braun et al., 2019; J. D. Neilson et al., 2009, 2014) tagging programs. Such highly migratory behavior mirrors a complex phylogeographic signature (Bremer et al., 2005) and genetic population structure (Kotoulas et al.,

2007) that challenge the stock management of the Regional Fisheries Management Organizations (RFMOs). In the Atlantic and Mediterranean regions, the International Commission for the Conservation of Atlantic Tunas (ICCAT) is the reference RFMO, which manages swordfish as three separate stocks, North Atlantic, South Atlantic and Mediterranean according to multiple lines of evidence including tagging (Braun et al., 2019), population genetics (Kotoulas et al., 2007; Righi, Splendiani, Fioravanti, Petetta, et al., 2020; Smith et al., 2015; Viñas et al., 2010) and parasite infections (Garcia et al., 2011). Although it is well established that the three areas correspond to distinct population, the boundaries and mixture between them are still a matter of debate (Smith et al., 2015). Furthermore, both the North Atlantic and Mediterranean stocks have suffered from overfishing periods in the last decades (ICCAT, 2017a; J. Neilson et al., 2013) leading to a demographic decrease of population size with unknown genetic consequences. For these reasons, the objectives of the present study were to 1) gain deeper insights into the genetic structure and genetic diversity at genomic level of the three swordfish stocks, the North Atlantic, the South Atlantic and the Mediterranean, 2) assess the stocks fitness and evolutionary potential to respond to environmental changes and 3) confirm the current spatial stock boundaries and degree of mixture. To pursue such objectives, we leveraged the power of *de novo* genome assembly and double digest restriction associated DNA (ddRAD) sequencing, a strategy that allows better genotyping performances and a genome-wide assessment of SNPs. The results obtained herein represent a set of information essential to delineate conservation units and to support a science-based decision-making process in the context of fishery management.

3.3 Materials and methods

Sampling activities

A total of 576 swordfish were sampled from 14 sampling areas in the Atlantic Ocean and Mediterranean Sea (Fig. 1). The management boundaries at 5°N, separating the North Atlantic stock from the South Atlantic stock, and at the Strait of Gibraltar, separating the North Atlantic stock from the Mediterranean stock, were initially used to assign localities to the corresponding three management units. All fish were collected between 2016 and 2020 by commercial fishing or research vessels using longlines in the framework of the International Commission for the Conservation of Atlantic Tunas (ICCAT) project “Swordfish Year Program”. For each specimen, a piece of the caudal fin or muscle tissue was collected, stored in absolute ethanol and kept at −20°C until DNA extraction for ddRAD analysis. A blood sample (1.5 ml) was collected from a single swordfish caught in the Adriatic Sea and kept at -20°C until DNA extraction for genome sequencing.

Figure 1 - Sampling from 14 sampling areas in the Atlantic Ocean and Mediterranean Sea.

Genome sequencing and assembly

DNA was extracted from the blood sample using the HMW DNA Extraction kit following the manufacturer's protocol. DNA concentration and integrity were analysed using NanoPhotometer® P-Class (Implen, Munich, Germany) and Gel Red™ staining of genomic DNA band on 1% gel, respectively. The DNA extracted was sequenced using a mixed strategy consisting of Nanopore (MinION) and Illumina technology (NovaSeq 6000). The quality of the Illumina paired-end reads was first analysed using FastQC (Andrews, 2010), then BBDuk (https://sourceforge.net/projects/bbmap/) was used to remove low quality bases (<Phred 30), adapters and short sequences (minimum length 35 bp). Genome size estimation was then performed using only high-quality reads with BBMap. Nanopore reads were corrected with the software NECAT (Chen et al., 2020) and then assembled with the software Canu (Koren et al., 2017) and wtdbg2 (Ruan & Li, 2020) by setting an expected genome size of 600 Mbp. The raw assemblies were corrected using the Illumina data performing three iterations of NTHITS (options -b 36 -k 40 -t 36 --outbloom --solid) and NTEDIT (-k 40 -z 1000 -d 3). The corrected assemblies were then merged with the software Quickmerge (Chakraborty et al., 2016) with the option -l 490000. The obtained merged assembly was further polished with five iterations of NTHITS/NTEDIT. The Illumina reads were mapped against the assembly with minimap2 (Li, 2018), then Platypus (Rimmer et al., 2014) was used to identify mismatches between the assembly and the reads (options --minReads=6 --nCPU=20 --minFlank=10 --trimReadFlank=10 --assemble=1 --assembleBadReads=1 --assemblerKmerSize=55 --assembleBrokenPairs=1 --minMapQual=30 --minBaseQual=30 --minPosterior=30). The high-quality variants (filter "PASS" and GT=1/1) were used to further polish the assembly with vcf-consensus (Danecek et al., 2011). Finally, RNA-seq reads previously obtained in our lab (Gioacchini et al., 2019) were mapped against the assembly with minimap2 with the option -x splice. The resulting mapping file was processed with Opossum (Oikkonen & Lise, 2017) (options --MinFlankEnd 10 --MinFlankStart 10 --SoftClipsExist True --ProperlyPaired False) and then Pilon (Walker et al., 2014) was used to perform an additional correction of the assembly (options --minqual 30 --minmq 30 --mindepth 6 --flank 0 --threads 20 --duplicates --fix bases --diploid --changes). Two iterations of mapping and Pilon corrections were performed. The software QUAST (Gurevich et al., 2013) was used to obtain the statistics of the assembly. As a final step, the genome scaffolds were anchored on the chromosome level assembly recently published by Wu et al. (2021) (Wu et al., 2021) using the software ragoo (Alonge et al., 2019) with default parameters. The unanchored scaffolds were joined to create a chromosome 0. The genes previously identified in the scaffolds were then transferred to the newly anchored genome using the software flo (Pracana et al., 2017).

Genome Annotation

Repeats were annotated on the final version of the genome using RepeatMasker. The annotation of the genes was performed with multiple steps in order to integrate different sources of information: 1) normalized RNA-seq reads from Gioacchini et al., (2019) were mapped against the genome with STAR version 020201 (Dobin et al., 2013) with the two-pass mode enabled. A reference guided transcriptome assembly was performed with Trinity v2.8.6 (Grabherr et al., 2011) with the options --SS_lib_type RF --jaccard_clip --full_cleanup --min_kmer_cov 2 --no_normalize_reads --genome_guided_max_intron 100000. The obtained transcripts were filtered to retain only those with a length higher than 500 bp 2) the software Maker (Cantarel et al., 2008) was run on the assembly providing the RepeatMasker annotation, the Trinity assembly and using zebrafish as model species for Augustus (Stanke & Morgenstern, 2005) to perform an initial step of annotation. The obtained genes were split into a training and test dataset to train a swordfish Augustus model with etraining and optimize_augustus.pl. 3) The output of STAR obtained by mapping the RNA-seq reads against the assembly was used with GeneMark (Besemer & Borodovsky, 2005) in order to obtain an HMM model for gene prediction. 4) A final Maker run was performed providing the assembled transcriptome, the RepeatMasker annotation, the Augustus model, the GeneMark model and a dataset of proteins from Lates calcarifer (ASM164080v1). The obtained GFF3 file was converted to GTF with gffread (Pracana et al., 2017) and then used as input with the RNA-seq BAM file for stringTie (Pertea et al., 2015) in order to annotate splicing isoforms and also annotate noncoding RNAs. The CDS features were added and final formatting of the GFF3/GTF files were done with the genometools program (Gremme et al., 2013). The functional annotation of the genes consisted in the prediction of long non-coding RNAs with PLEK (A. Li et al., 2014) whereas descriptions and Gene Ontology annotations were attributed with the software Pannzer2 (Törönen et al., 2018) setting a minimum query coverage or a minimum subject coverage of 0.4 and a minimum alignment length of 50. The KEGG annotations were obtained using the KEGG annotation server and selecting the following as species: hsa, mmu, rno, dre, cel, ecu, nme, bsu, mtu, syn, aae, ape, lcf, npr, lcm, sdu, tru, ipu, srx, xma, onl, cvg. Genome completeness was evaluated with BUSCO (Simão et al., 2015) using the Actinopterygii, Eukaryote and Metazoa databases. Finally, to further assess the quality and consistency of the genome assembly, orthofinder (Emms & Kelly, 2015) was applied with default parameters using 19 fish genomes downloaded from Ensembl (www.ensembl.org) to identify groups of orthologous genes. The list of fish species used for the comparison can be accessed in the Supplementary Material 3.

ddRAD sequencing

Genomic DNA was extracted from ~25mg of tissue using Quick-DNA™ Miniprep Kit (Zymo Research, USA) following the manufacturers' protocols. Upon extraction, purity and quantity of total DNA were checked using the nanophotometer P330 (Implen, Germany). Then, DNA samples were sequenced at the IGA Technology Service (Udine, Italy) using double digest restriction-site associated DNA sequencing (ddRAD). Libraries were constructed using *Sph*I and *MboI* restriction enzymes and sequenced with the Illumina NovaSeq6000. A total of 719,722,497 paired reads were sequenced. The raw reads were trimmed to remove low quality bases and adapters using BBDuk (Bushnell, et al., 2017), using a minimum PHRED score of 35 and a minimum reads length of 35 bp. The trimmed reads were mapped to our genome assembly using the MINIMAP2 program (Li, 2018). The BAM files sorted by coordinates were analyzed with SAMBAMBA (Tarasov et al., 2015) to remove duplicates. PICARD (https://broadinstitute.github.io/picard/command-line-overview.html#MarkDuplicates) was used to add the read group to each BAM obtained from each sample. The variant calling was done using the Platypus (Rimmer et al., 2014) program with the options to get only variants supported by at least 4 reads, ignoring the first and last 10 bases of the reads. Only reads with MAPQ> 10 and bases with a quality of at least 30 were considered. The BAMs were processed all together to obtain a single VCF with all the samples. The VCF obtained was filtered to leave only SNPs with the filter PASS (using column 7 of the VCF). VCFtools (Danecek et al., 2011) was then used to apply various filters to the VCF. All the positions where the missing percentage was higher than 10% and the mean depth was lower than 4x were not considered and the outliers were removed. The final file filtered for MAF> = 5 % was obtained with the option –maf 0.05 and removing the samples with more than 10% of missing values.

Genetic variation

Genetic variation among stocks and 14 sampling areas was investigated. A Principal Component Analysis (PCA) was performed by first generating a Provesti-distance (P-distance) matrix with the *bitwise.dist* function included in the R package *poppr* (Kamvar et al., 2014) with the option "*missing_match* = *F*" and then using the built-in function *prcomp* from R. The P-distance matrices were generated with the VCF2D is algorithm (https://github.com/BGI-shenzhen/VCF2Dis) using default parameters. Then, the obtained matrices were visualized as heatmaps using ClustVis (Metsalu & Vilo, 2015) without row centering and scaling. The fixation index (F_{st}) was analyzed using *STAMPP* 1.5. method Weir and Cockerham (Pembleton et al., 2013). The allelic richness (total and mean) was calculated using the R package *PopGenReport* (Adamack & Gruber, 2014), while the heterozigosity (observed and expected) and F_{is} index were calculated using *Hierfstat* (Goudet, 2005). The allelic richness values for each variant were exported as a BED file which was then intersected with a second BED file containing 50 Kbp windows generated across the entire genome, using BEDTOOLS. The average allelic richness per window was then calculated using BEDTOOLS and used to generate plots with ggplot2 (Wickham, 2014). The BED file containing the allelic richness per variant, was also intersected with the coordinates of the annotated genes in the genome using BEDTOOLS, which was also used to extract the average allelic richness, the number of variants per gene and the standard deviation. For each gene a t-Test was performed in order to identify genes with a significant difference in allelic richness among the sampling areas and the p-values were corrected for multiple testing using the Benjamini-Hochberg correction in R. Finally, in order to identify genes with the highest variability across the sampled areas, the coefficient of variation (CV) for each marker was calculated across all the samples, then the corresponding Z-scores were calculated and using a z-test the scores were converted into p-values using the *stats* package which were corrected for multiple testing using the Benjamini-Hochberg correction in R (R core team 2021).

Genetic population structure analysis

Genetic population structure among sampling groups was explored using the Bayesian genotypic clustering implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) with admixture model. The number of hypothetical clusters tested (K) was set from 1 to 5. Markov chain Monte Carlo with 100,000 iterations with a burn-in period of 10,000 were carried for each K-value. The ΔK based on the Evanno method (Evanno et al., 2005) was applied to determine the optimal number of clusters using Structure Harvester (Earl & vonHoldt, 2012). The results were visualized using Distruct v2.3 (Chhatre, 2018). A spatial Bayesian analysis was performed with the GeneLand v4.0.9 algorithm (Guillot et al., 2005). The VCF file was converted to the required format using PGDspider v2.1.1.5 (Lischer & Excoffier, 2012). Since GeneLand was not able to analyze the entire dataset of SNPs, we generated lists of 100 randomly sampled variants for the pairwise analyses MED vs ATL-N and MED vs ATL-S. The number of variants was selected performing the analysis on random subsets of 20, 50, 100 and 250 variants, until the results did not change. For the ATL-N vs ATL-S analysis, the top 250 discriminating variants were selected performing a Kruskal Test on the variants allele frequencies. For each comparison, the analysis was performed using the MCMC command with the following settings: rate.max = total number of samples across the two analyzed populations; delta.coord = 1; npopmax=2; nb.nuclei.max = 3*(total number of samples across the two analyzed populations); nit = 100000; thinning = 100; freq.model = "Correlated"; varnpop = TRUE; spatial = T; npopmin = 2. Postprocessing was done applying a burning rate of 200.

3.4 Results

Genome assembly and assessment

Read assembly resulted in a haplotype genome of 687.5 Mb and an average GC content of 40.7%. A total of 4,493 scaffolds with an N50 score of 9.37 Mb were obtained. The longest scaffold was 50.25 Mb. The evaluation of the genome using BUSCO confirmed that between 87.4 and 92.5% of complete and single copy orthologs were present (Tab. 1).

Complete and single copy	Complete and duplicated	Fragmented	Missing	Database
3354	44	65	177	Actinopterygii
223	4	7	21	Eukaryote
883	24	13	34	Metazoa

Table 1 - Genome completeness according to BUSCO analysis using different databases.

Furthermore, between 1.2% and 2.5% of complete duplicated, between 1.3% and 2.7 fragmented and between 3.5% and 8.2% missing orthologues were identified (Fig. 2).

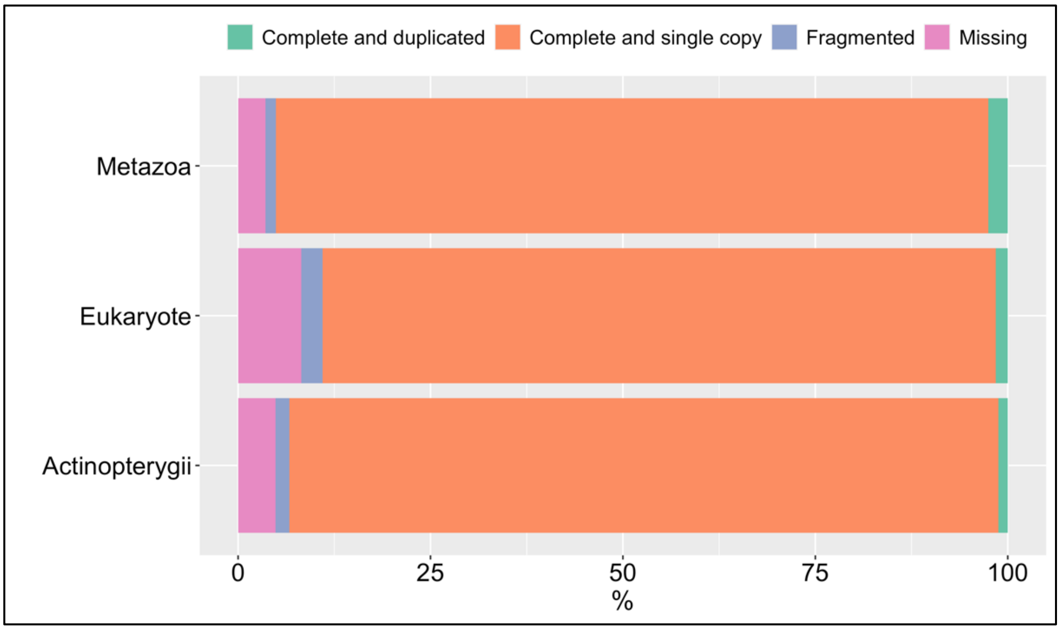
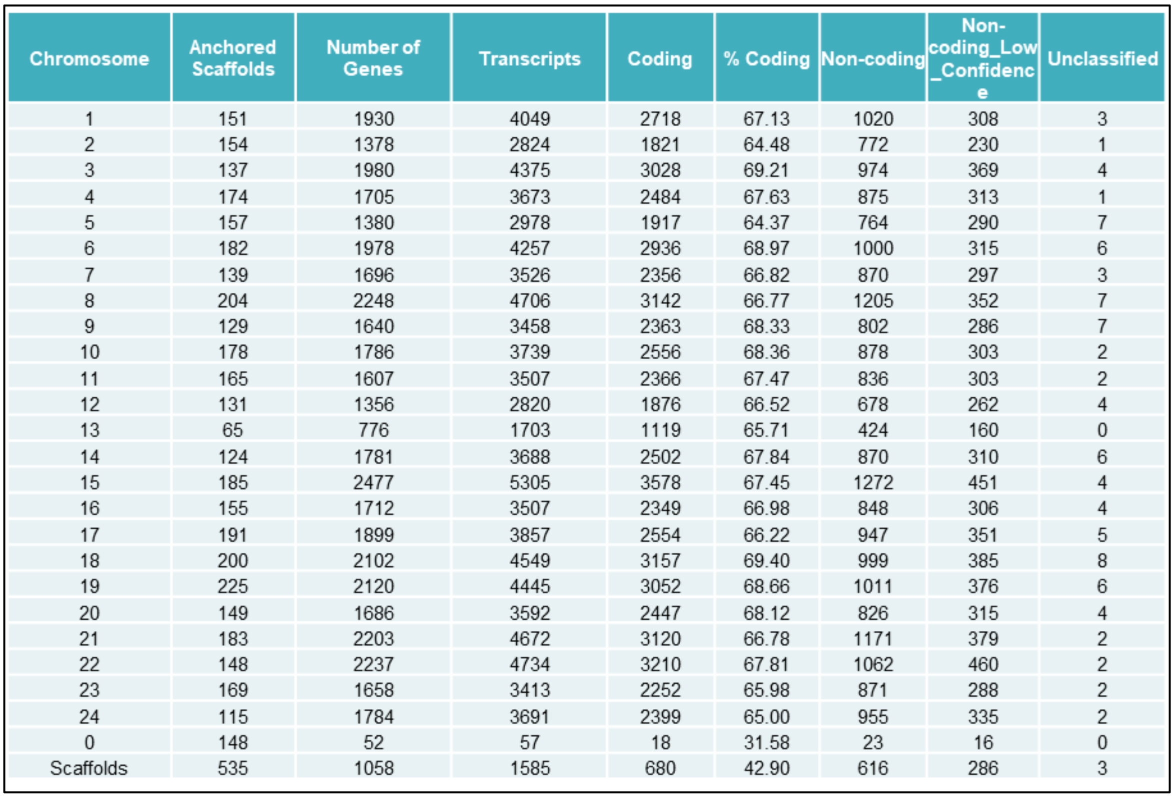


Figure 2 - Graphical representation of genome completeness according to BUSCO analysis using different databases.

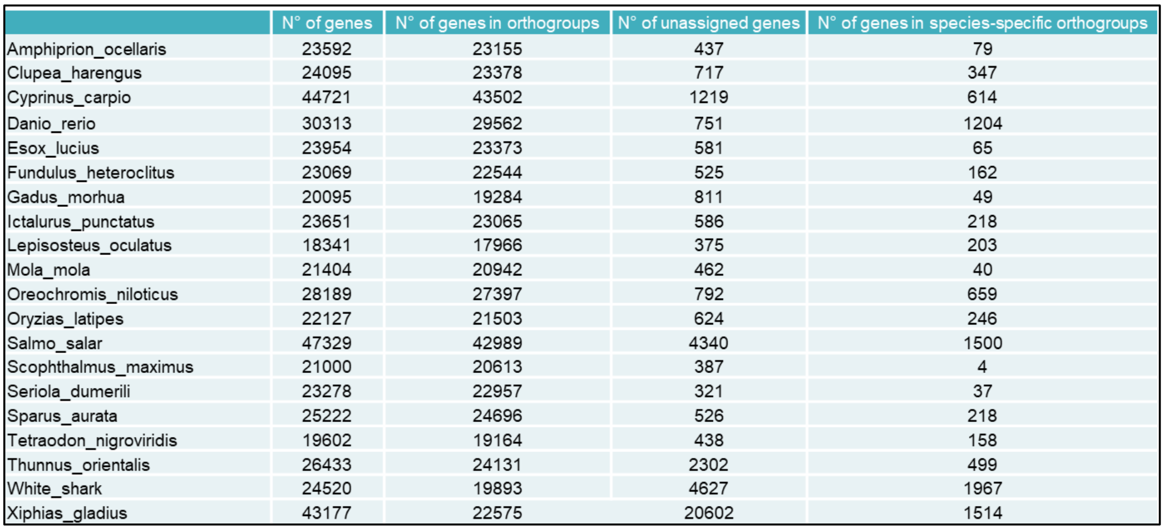
A total of 21,660 protein-coding and 22,569 high-confidence non-coding genes were identified in the swordfish genome using the experimental evidence from the RNA-seq. Interspersed reads represented 4.17% of the genome, with DNA transposon and retroelements accounting for 2.61% and 1.54%, respectively. A total of 148 scaffolds could not be anchored to the genome published by Wu et al. (2021) and therefore were grouped in a “chromosome 0”. Furthermore, 535 scaffolds were not associated with any of the chromosomes present in such assembly. The detailed metrics of the chromosome content and anchoring process can be found in Figure 3.



Chromosome	Anchored Scaffolds	Number of Genes	Transcripts	Coding	% Coding	Non-coding	Non-coding_Low_Confidence	Unclassified
1	151	1930	4049	2718	67.13	1020	308	3
2	154	1378	2824	1821	64.48	772	230	1
3	137	1980	4375	3028	69.21	974	369	4
4	174	1705	3673	2484	67.63	875	313	1
5	157	1380	2978	1917	64.37	764	290	7
6	182	1978	4257	2936	68.97	1000	315	6
7	139	1696	3526	2356	66.82	870	297	3
8	204	2248	4706	3142	66.77	1205	352	7
9	129	1640	3458	2363	68.33	802	286	7
10	178	1786	3739	2556	68.36	878	303	2
11	165	1607	3507	2366	67.47	836	303	2
12	131	1356	2820	1876	66.52	678	262	4
13	65	776	1703	1119	65.71	424	160	0
14	124	1781	3688	2502	67.84	870	310	6
15	185	2477	5305	3578	67.45	1272	451	4
16	155	1712	3507	2349	66.98	848	306	4
17	191	1899	3857	2554	66.22	947	351	5
18	200	2102	4549	3157	69.40	999	385	8
19	225	2120	4445	3052	68.66	1011	376	6
20	149	1686	3592	2447	68.12	826	315	4
21	183	2203	4672	3120	66.78	1171	379	2
22	148	2237	4734	3210	67.81	1062	460	2
23	169	1658	3413	2252	65.98	871	288	2
24	115	1784	3691	2399	65.00	955	335	2
0	148	52	57	18	31.58	23	16	0
Scaffolds	535	1058	1585	680	42.90	616	286	3

Figure 3 - The detailed metrics of the chromosome content and anchoring process.

A total of 22,575 (52.3%) swordfish genes were grouped in shared orthologous groups of genes while 20,602 (47.7%) genes were unassigned. A large fraction (15,587, 75%) of these genes were non-coding and 4974 (24%) were protein-coding genes. A total of 1514 (3.5%) of swordfish genes were found being swordfish specific. The complete results of the analysis of orthologous groups of genes can be found in Fig 4.



	N° of genes	N° of genes in orthogroups	N° of unassigned genes	N° of genes in species-specific orthogroups
Amphiprion_ocellaris	23592	23155	437	79
Clupea_harengus	24095	23378	717	347
Cyprinus_carpio	44721	43502	1219	614
Danio_rerio	30313	29562	751	1204
Esox_lucius	23954	23373	581	65
Fundulus_heteroclitus	23069	22544	525	162
Gadus_morhua	20095	19284	811	49
Ictalurus_punctatus	23651	23065	586	218
Lepisosteus_oculatus	18341	17966	375	203
Mola_mola	21404	20942	462	40
Oreochromis_niloticus	28189	27397	792	659
Oryzias_latipes	22127	21503	624	246
Salmo_salar	47329	42989	4340	1500
Scophthalmus_maximus	21000	20613	387	4
Seriola_dumerili	23278	22957	321	37
Sparus_aurata	25222	24696	526	218
Tetraodon_nigroviridis	19602	19164	438	158
Thunnus_orientalis	26433	24131	2302	499
White_shark	24520	19893	4627	1967
Xiphias_gladius	43177	22575	20602	1514

Figure 4 - Analysis of orthologous groups of genes

Population genetics

After the filtering procedure, 57 samples were discarded. The sequencing of RADs libraries of 519 genotypes produced a total of 719,722,497 raw reads, corresponding to an average of 1,386,748 paired reads per sample, which resulted in a total of 26,324 SNPs identified using the reference genome to guide the process of SNPs calling (Fig. 4).

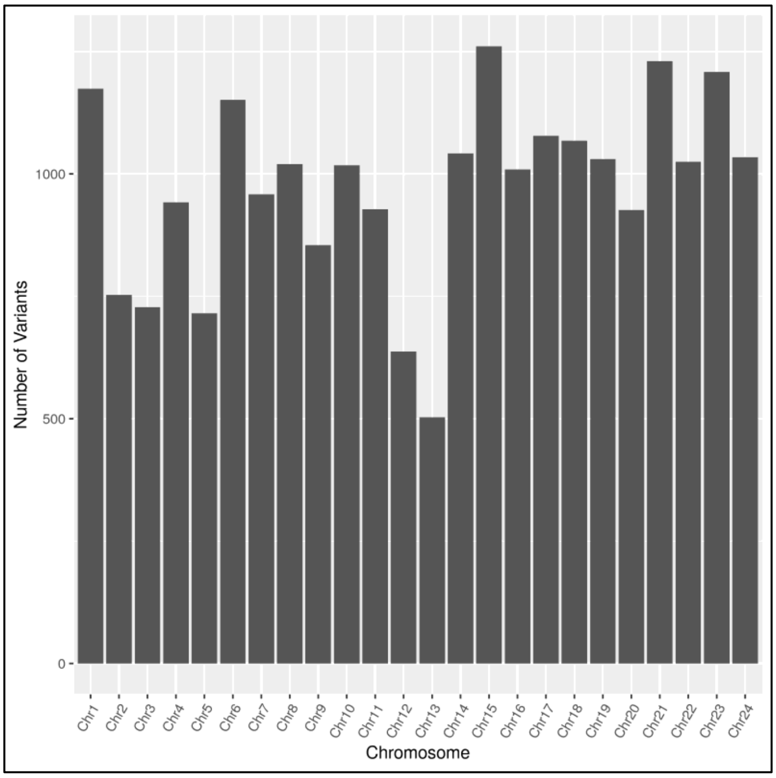


Figure 4 - A total of SNPs identified using the reference genome to guide the process of SNPs calling for each chromosome.

To evaluate the overall genetic structure, a principal component analysis (PCA) was performed considering all the 519 selected samples. PC1 explained 66.02% of variance and well separated the samples in two main clusters, one corresponding to the majority of the samples collected from the Atlantic (*n*=370), and another corresponding to all the samples collected in the Mediterranean (*n*=149) in addition to 11 swordfish collected in the North Atlantic (Fig. 5).

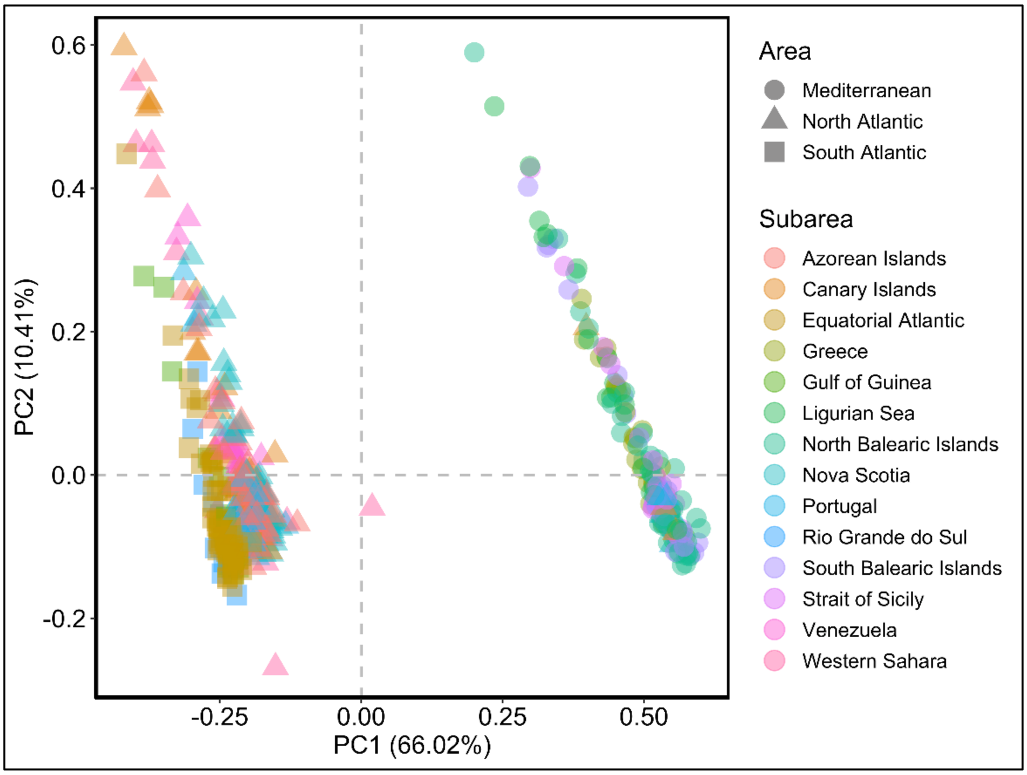


Figure 5 - Principal components analysis of SNPs for swordfish from 3 stocks and 14 sampling areas. Each point represents one individual fish. Colour codes correspond to sampling area and shape code correspond to 3 stocks.

Among those collected in the Atlantic but clustering with the Mediterranean samples, 5 were caught in the Canary Islands and 6 in Portugal. Furthermore, the probability of catching Mediterranean specimens in Portugal was around 14% and in Canary Islands was around 10%. One of the 5 specimens caught in Canary Islands area with Mediterranean genotype, was caught at 27°N, very far south of the Strait of Gibraltar, the assumed stock boundary. On the contrary, no differences in clustering were observed in the PCA analysis between North and South Atlantic stocks. The heatmap plot (Fig. 6), based on the P-distance matrix for all specimens, showed a higher genetic diversity between Mediterranean and Atlantic stocks, while a weak genetic differentiation was seen between North and South Atlantic stocks.

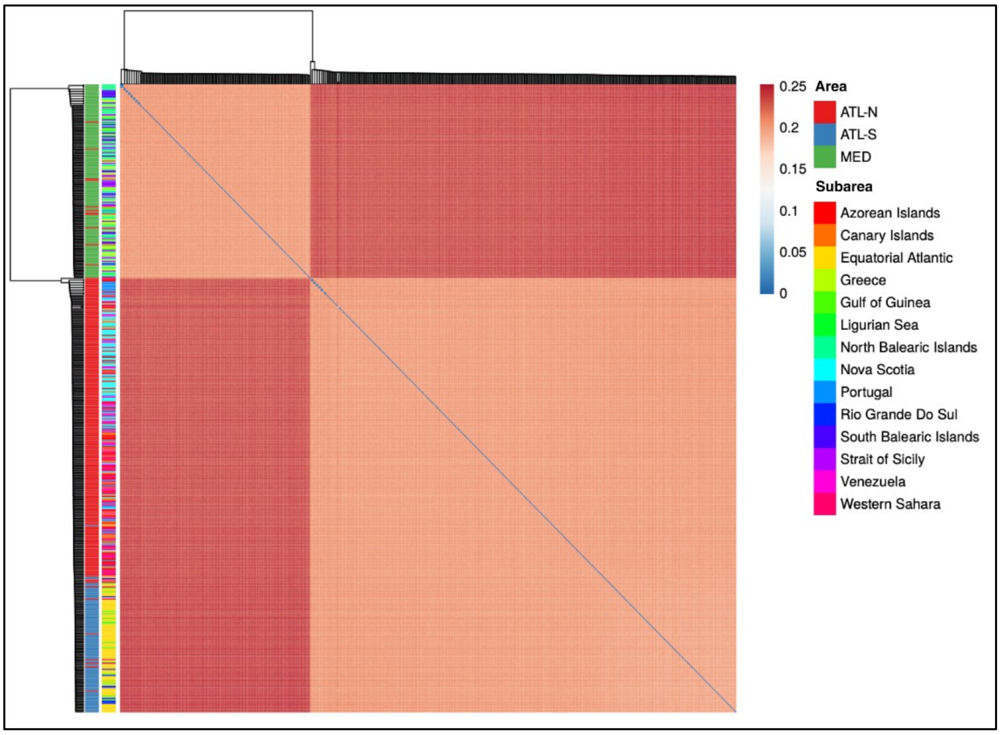


Figure 6 - A heat map plotted using the R package ClustVis from a genetic Provesti distance matrix using all SNPs. The genetic distance matrix was generated with the VCF2Dis algorithm. Blue indicates identical genotypes, whereas high genetic dissimilarity is displayed in red.

11 specimens caught in Portugal and Canary Islands sampling areas were from the Mediterranean stock, with those being the same specimens that had been identified by the PCA analysis. In addition, 9 specimens caught in the Equatorial Atlantic area at latitudes higher than the 5°N management boundary, and off the Western Sahara at latitudes of 20ºN, clustered with the South Atlantic specimens. The pairwise Fixation index (F_{st}) among stocks is shown in Table 2.

Stock	ATL-N	ATL-S	MED
ATL-N	NA	NA	NA
ATL-S	**0.00960785**	NA	NA
MED	**0.08585356**	**0.1104412**	NA

Significant differences (p-value < 0.05) are displayed in bold

Table 2 - The pairwise FST (Fixation index) among the 3 stocks. Significant differences (*p-value* < 0.05) are displayed in bold.

The highest genetic difference was found between specimens from the Mediterranean and the South Atlantic (F_{st}=0.1104), the lowest between North Atlantic and South Atlantic (F_{st}=0.0096). Comparison of sampling areas provides further nuance in these genetic spatial patterns (Table 3). Among 6 North Atlantic sampling areas, the lowest value was shown in the comparison between Nova Scotia and Venezuela (F_{st}=0.0004), while the highest between Portugal and Nova Scotia (F_{st}=0.0051), showing a slight genetic differentiation between east and west north Atlantic stock. Among 3 South Atlantic sampling areas the lowest value was shown in the comparison between Gulf of Guinea and Equatorial Atlantic (F_{st}=0.00005), while the highest between Rio Grande Do Sul and Gulf of Guinea (F_{st}=0.0041), confirming also for the south Atlantic stock a slight genetic differentiation between east and west specimens. Among 5 Mediterranean sampling areas, the lowest value is shown in the comparison between Greece and the Ligurian Sea (F_{st}=0.0004), while a higher value was observed between Greece and South Balearic Islands (F_{st}=0.0035).

Sampling Areas	Gulf of Guinea	Equatorial Atlantic	Rio Grande Do Sul	Venezuela	Nova Scotia	Azorean Islands	Western Sahara	Canary Islands	Portugal	Balearic Islands-N	Balearic Islands-S	Ligurian Sea	Strait of Sicily	Greece
Gulf of Guinea	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Equatorial Atlantic	0.000046	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Rio Grande Do Sul	**0.004071**	**0.003840**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Venezuela	**0.011342**	**0.011285**	**0.017029**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nova Scotia	**0.014461**	**0.014518**	**0.019938**	**0.000391**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Azorean Islands	**0.010020**	**0.010003**	**0.015128**	**0.000579**	**0.001160**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Western Sahara	**0.006120**	**0.005967**	**0.011352**	**0.001578**	**0.003013**	**0.000902**	NA	NA	NA	NA	NA	NA	NA	NA
Canary Islands	**0.010170**	**0.010707**	**0.015061**	**0.001266**	**0.002887**	**0.001637**	**0.001976**	NA	NA	NA	NA	NA	NA	NA
Portugal	**0.011347**	**0.012544**	**0.016787**	**0.003220**	**0.005066**	**0.003574**	**0.003493**	**0.001216**	NA	NA	NA	NA	NA	NA
Balearic Islands-N	**0.110251**	**0.111956**	**0.116885**	**0.092325**	**0.092886**	**0.094012**	**0.095649**	**0.073452**	**0.072385**	NA	NA	NA	NA	NA
Balearic Islands-S	**0.111108**	**0.112717**	**0.117197**	**0.093524**	**0.093791**	**0.094918**	**0.096322**	**0.073693**	**0.072736**	**0.003120**	NA	NA	NA	NA
Ligurian Sea	**0.109926**	**0.111404**	**0.116677**	**0.091948**	**0.092582**	**0.093286**	**0.094885**	**0.071691**	**0.071193**	**0.002135**	**0.003081**	NA	NA	NA
Strait of Sicily	**0.110432**	**0.112129**	**0.116875**	**0.092504**	**0.092750**	**0.093899**	**0.095352**	**0.072597**	**0.071375**	**0.001692**	**0.002474**	**0.001864**	NA	NA
Greece	**0.110786**	**0.112423**	**0.117730**	**0.093345**	**0.093598**	**0.094647**	**0.096238**	**0.073361**	**0.072623**	**0.002714**	**0.003541**	**0.000437**	**0.002432**	NA

Significant differences (p-value < 0.05) are displayed in bold

Table 3 - The pairwise FST (Fixation index) among the 14 sampling areas. Significant differences (p-value < 0.05) are displayed in bold.

For each stock and sampling area, the observed and expected heterozygosity (H_o and H_e), the inbreeding index (F_{is}), and the mean and total allelic richness were calculated. Within each of the three stocks, H_o values were always lower than H_e values (Table 4).

Stock	Ho	Hs	Fis	TotalRichness	MeanRichness
ATL-N	0.229	0.239	0.047	52712.272	2.002
ATL-S	0.225	0.233	0.033	52110.544	1.980
MED	0.236	0.243	0.029	50128.632	1.904

Table 4 - Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), Inbreeding Coefficient (F_{is}), Total Allelic Richness, Mean Allelic Richness among stocks

The H_o values were similar among the three stocks, however samples from the South Atlantic showed the lowest value of H_o (0.225) with F_{is}=0.033 and those from the Mediterranean Sea showed the highest H_o (0.236) with the lower F_{is} (0.028). Spatial patterns in heterozygosity were confirmed when analysing the 14 sampling areas (Table 5).

Sampling Areas	Ho	Hs	Fis	TotalRichness	MeanRichness
Gulf of Guinea	0.224008145	0.233514933	0.031114399	42060.60964	1.597804651
Equatorial Atlantic	0.227317007	0.233623074	0.027935908	42443.72162	1.612358366
Rio Grande Do Sul	0.223649255	0.231000099	0.02252478	41603.89012	1.580454723
Venezuela	0.225630903	0.23542206	0.034486115	42324.51908	1.607830082
Nova Scotia	0.227704346	0.235863566	0.032784192	42585.48604	1.617743734
Azorean Islands	0.228591654	0.236763888	0.032531219	42623.86027	1.619201499
Western Sahara	0.231610618	0.237946596	0.027443149	42728.2161	1.623165784
Canary Islands	0.229108479	0.243128358	0.056633198	43158.53127	1.63951266
Portugal	0.228714189	0.2431012	0.058278547	43150.86627	1.639221481
North Balearic Islands	0.235667516	0.242784489	0.026899393	42299.94508	1.606896561
South Balearic Islands	0.236063307	0.242580364	0.022597547	42187.56654	1.602627509
Ligurian Sea	0.238848408	0.243558186	0.01594905	42168.22542	1.601892776
Strait of Sicily	0.236167266	0.242491776	0.023164601	42160.46023	1.60159779
Greece	0.234561742	0.242228411	0.028547165	42210.72645	1.603507311

Table 4 - Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), Inbreeding Coefficient (F_{is}), Total Allelic Richness, Mean Allelic Richness among 14 sampling areas.

Canary Islands and Portugal showed a higher value of H_o and F_{is} with respect the other north Atlantic sampling areas which may be due to the presence of the 11 specimens caught in these areas that belong to the Mediterranean stock. To confirm this hypothesis, H_o and F_{is} were calculated without considering these 11 specimens. As expected, with that exclusion, the Canary Islands and Portugal showed a reduction of H_o and F_{is} values to comparable levels observed in the other North Atlantic sampling areas (Table 6).

Sampling Areas	Ho	Hs	Fis	TotalRichness	MeanRichness
Gulf of Guinea	0.224008	0.233515	0.031114	42060.61	1.597805
Equatorial Atlantic	0.227317	0.233623	0.027936	42443.72	1.612358
Rio Grande Do Sul	0.223649	0.231	0.022525	41603.89	1.580455
Venezuela	0.225631	0.235422	0.034486	42324.52	1.60783
Nova Scotia	0.227704	0.235864	0.032784	42585.49	1.617744
Azorean Islands	0.228592	0.236764	0.032531	42623.86	1.619201
Western Sahara	0.231611	0.237947	0.027443	42728.22	1.623166
Canary Islands	0.228629	0.237137	0.032756	42605.59	1.618508
Portugal	0.227449	0.235948	0.033477	42507.32	1.614774
North Balearic Islands	0.235668	0.242784	0.026899	42299.95	1.606897
South Balearic Islands	0.236063	0.24258	0.022598	42187.57	1.602628
Ligurian Sea	0.238848	0.243558	0.015949	42168.23	1.601893
Strait of Sicily	0.236167	0.242492	0.023165	42160.46	1.601598
Greece	0.234562	0.242228	0.028547	42210.73	1.603507

Table 5 - Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), Inbreeding Coefficient (F_{is}), Total Allelic Richness, Mean Allelic Richness among 14 sampling areas without the 11 specimens caught in North Atlantic clustered with Mediterranean specimens.

However, when considering both mean and total allelic richness, a substantially different scenario was observed. The Mediterranean stock showed the lowest value of mean allelic richness (1.904) with respect to both North (2.002) and South (1.979) Atlantic stocks (Table 4). Interestingly, an uneven distribution of allelic richness at the chromosome-level was identified which reflected specific trends on certain chromosomes linked to different stocks (Fig. 7).

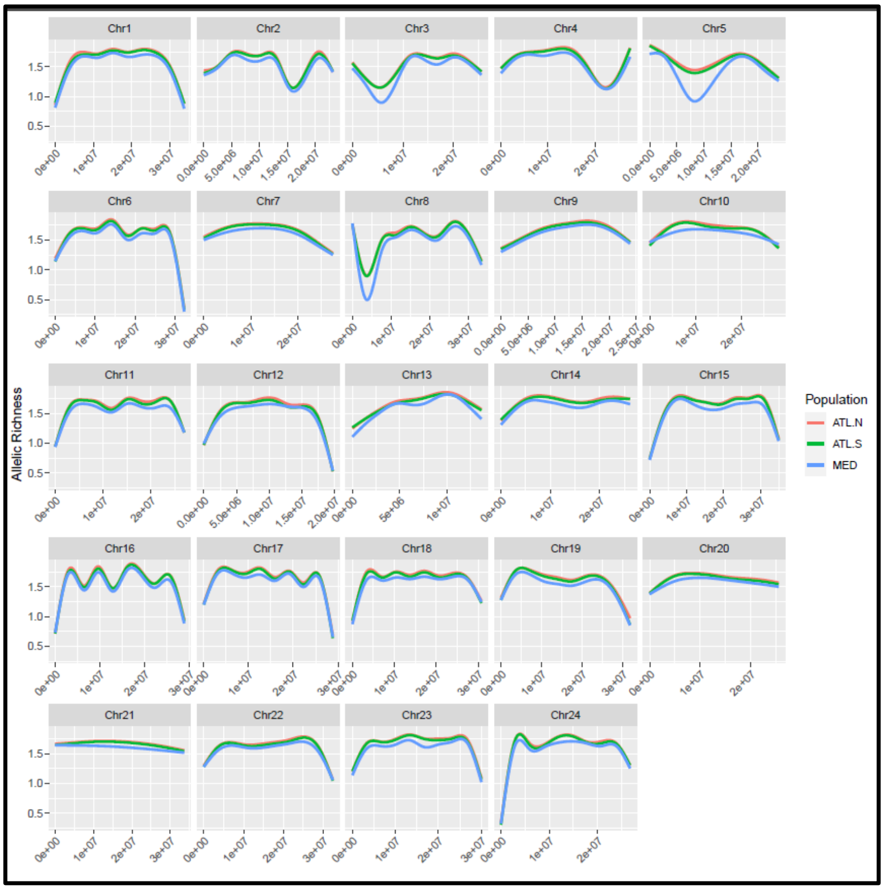


Figure 7 - Distribution of allelic richness at the chromosome-level.

Indeed, lower allelic richness was found in most chromosomes when Mediterranean stock was compared with Atlantic ones. In particular, the Mediterranean stock showed a lower allelic richness in chromosome 3, 5 and 8 (Figure 7). This result was reflective of trends of allelic richness also at the gene-level among 3 stocks and 14 sampling areas (Figure 8A). Noteworthy, Portugal and Canary Islands show an intermediate trend between the rest of the North Atlantic areas and the Mediterranean ones.

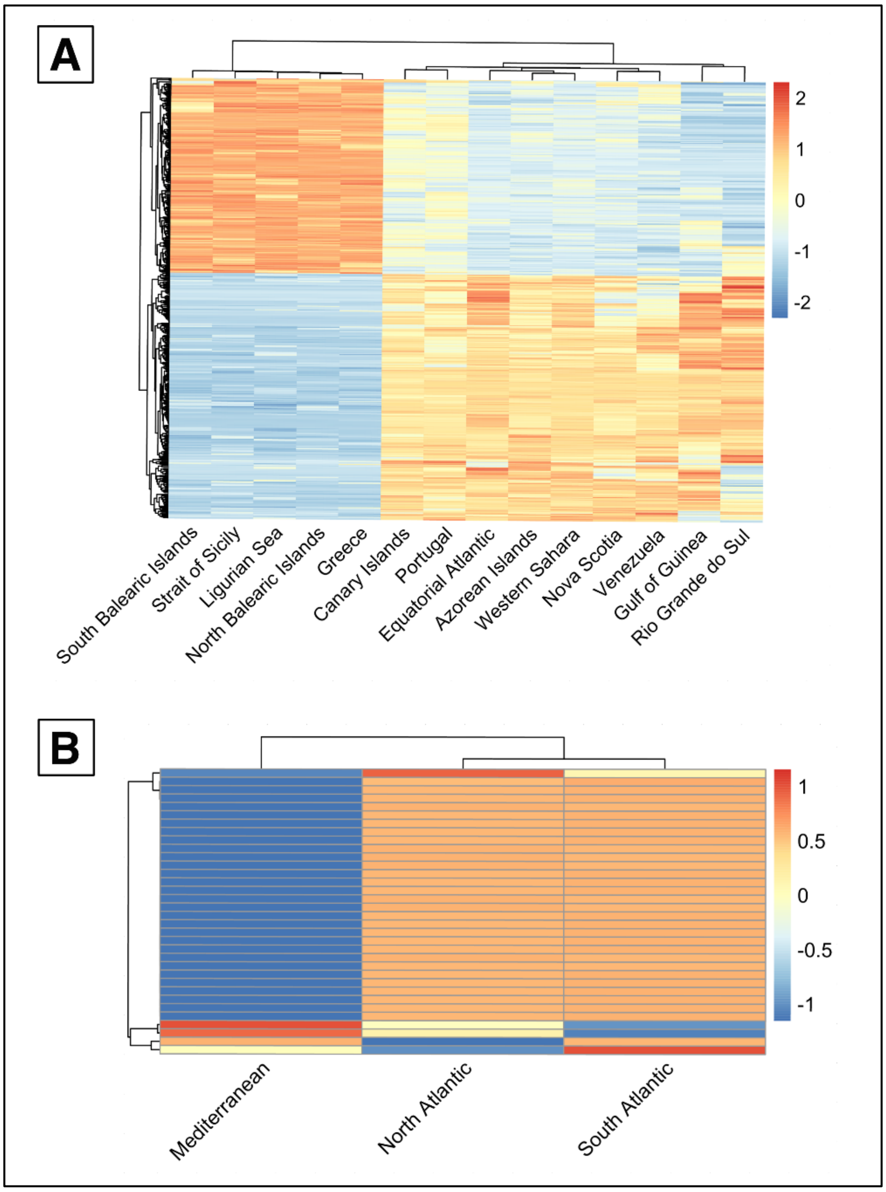


Figure 8 - A) Trends of allelic richness also at the gene-level among 3 stocks and 14 sampling areas; B) Genes with a different allelic richness (FDR<0.05) in at least one pair-wise comparison among North Atlantic, South Atlantic, and Mediterranean

This prompted us to investigate whether the distribution of allelic richness in the genome mirrored specific genes and biological functions. Such approach revealed a total of 47 genes with a different allelic richness (FDR<0.05) in at least one pair-wise comparison among North Atlantic, South Atlantic, and Mediterranean (Figure 8B). In such comparisons, a total of 40 genes were identified between North Atlantic and Mediterranean, 35 genes between South Atlantic and Mediterranean and only 1 gene between North and South Atlantic. Among these genes, 5 showed a significantly higher allelic richness in both North and South Atlantic stocks with respect to the Mediterranean. The 5 genes of notable biological importance are: the *aryl hydrocarbon receptor nuclear translocator-like protein 1* (*arntl*), involved in detoxification pathways (Carney et al., 2006), the *complement* *component 1, q subcomponent-like 3a* (*c1ql3a*) involved in the innate immune response (Ghai et al., 2007), the thiamine pyrophosphokinase (*tpk1*) and the cubilin (*cubn*) involved in vitamins up-take and metabolism (Calduch-Giner et al., 2016; Harder et al., 2018) and the 5-hydroxytryptamine receptor 1 (*htr1*), involved in neuroendocrine signaling (Winberg & Thörnqvist, 2016).

Population structure analysis

The genotypic clusters estimated by STRUCTURE supported the patterns shown by the PCA, pairwise F_{st}, and p-distance matrix results. The ΔK analysis revealed a high magnitude corresponding to K=2. The distribution of the two allelic frequencies clearly reflected the Mediterranean and the Atlantic stocks (Figure 9).

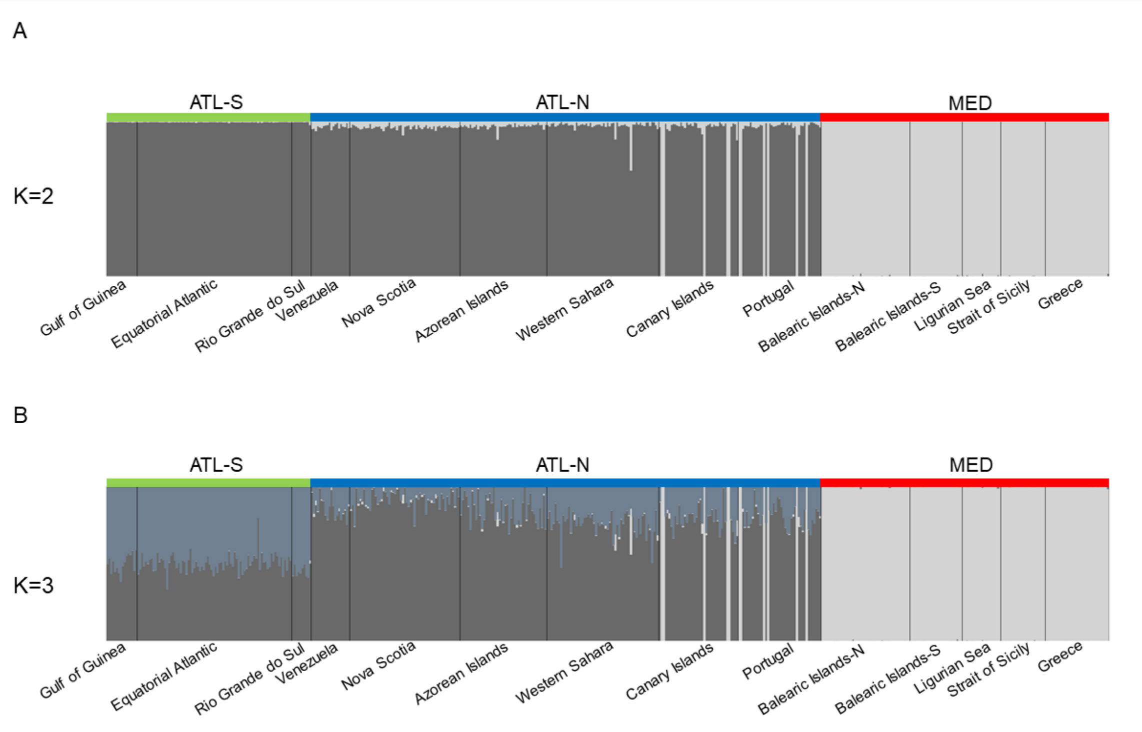


Figure 9 - Structure cluster assignment for K = 2 and K = 3 based on SNPs. Each genotype is one vertical bar, populations are separated by black lines and cluster assignment is indicated by grey, dark grey and blue colors.

Interestingly, 11 specimens caught in the North Atlantic showed a Mediterranean genotype and they are the same previously identified in the PCA and P-distance matrix analysis. Allelic frequencies characteristic of the Mediterranean stock were also found in all North Atlantic specimens with values ranging from 2.4% to 15.8% of allelic frequencies, but not in the South Atlantic ones. K=3 showed 3 clusters of allelic frequencies: one was attributable to Mediterranean stock; the other two frequencies were attributable to both North and South Atlantic stocks but with a different percentage. In particular, a gradient of allelic frequencies from North to South Atlantic was found, highlighting a population substructure of Atlantic Ocean. Interestingly, swordfish from North-East Atlantic sampling areas (Canary Islands, Azorean Islands, Portugal and Western Sahara), showed a mixing genotype containing all the three allelic frequencies. Once again, South Atlantic specimens did not show Mediterranean allelic frequency. The pairwise spatial Bayesian analyses were performed in GeneLand. The pairwise analysis between North Atlantic and Mediterranean stocks showed two cluster (Supplementary material 8A). Six specimens belonging to Portugal and 5 belonging to Canary Islands were assigned to cluster 2 with high posterior probabilities (≈0.9). The pairwise analysis between South Atlantic and Mediterranean stocks showed two distinctly clusters (Supplementary material 8B). Finally, the pairwise spatial analysis between North and South Atlantic stocks showed a unique cluster with no differences (Fig. 10).

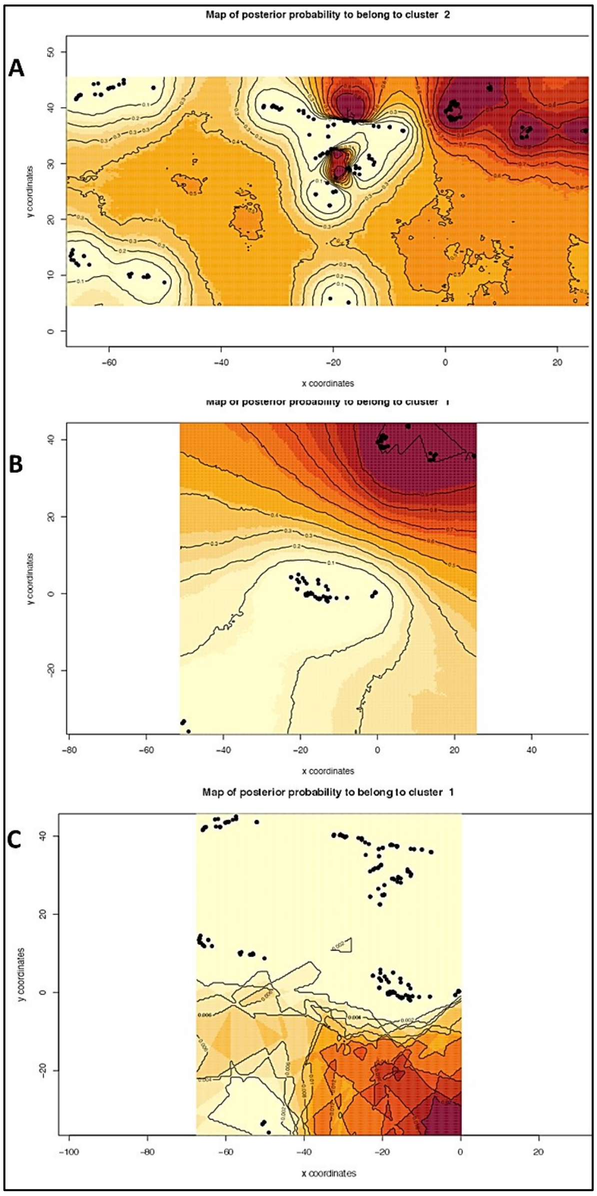


Figure 10 - Maps of posterior probabilities of population membership at K = 2 were inferred using GENELAND.

3.5 Discussion

The increased power of novel genotyping techniques allows the detection of more subtle, often previously hidden, genetic structures within many marine populations (Maroso et al., 2018; Saha et al., 2021). In this study, a genome-wide SNPs analysis combined with a genome assembly was applied for the first time to better describe the genetic structure of swordfish stocks from Atlantic Ocean and the Mediterranean Sea and to explore their genomic landscape. Our results, based on different multivariate and individual-based analyses of 26,324 SNPs, showed evidence of strong genetic differentiation between Mediterranean and Atlantic stocks, confirming previous studies based on mtDNA and SSRs variability (Alvarado Bremer et al., 2005; Kotoulas et al., 2007; Smith et al., 2015). In this study, the coupling of ddRAD sequencing with the genome assembly were able to both highlight the complex genetic structure and to characterize the genetic diversity among stocks as previously done for other marine species (Maroso et al., 2018; Vendrami et al., 2019). The role of genetic diversity on both individual fitness and evolutionary potential of a species is well-known (Allendorf et al., 2010; Eizaguirre & Baltazar-Soares, 2014; Frankham, 2003, 2010a, 2010b; Oldenbroek, 2017; Reed & Frankham, 2003). Genetic diversity reflects past evolutionary responses to environmental conditions and, at the same time, corresponds to the potentiality of a species to adapt to environmental challenges. Therefore, temporal and spatial monitoring of genetic diversity become essential within conservation strategies (Steffen et al., 2015). It is therefore important to identify appropriate genetic metrics to monitor genetic diversity of a stock. Genetic diversity is usually measured by the observed or expected heterozygosity (H_o and H_e) and by the number of alleles per locus for multiallelic markers or allelic richness (Greenbaum et al., 2014). These two measures of genetic diversity are complementary because whereas the former is directly related to genetic variance for quantitative traits and, therefore, to the short-term response to selection and adaptation for these traits (Falconer and Mackay, 1996), the latter is more sensitive to population bottlenecks (Leberg, 1998; Luikart et al., 1998), being thus useful to monitor the long-term response to natural and artificial selection (Caballero & García-Dorado, 2013; Hill & Rasbash, 1986; James, 1971; Medugorac et al., 2011; Vilas et al., 2015; Wilson et al., 2009). While not all variation is related to the adaptive potential, clearly no standing variation exists if no allelic richness exists. Moreover, there is evidence that high allelic richness, even of merely neutral alleles, increases evolvability by making a larger fraction of the genotypic space accessible by fewer mutational events (Wagner et al., 2008). Allelic richness is, therefore, a strong indicator for the evolutionary potential of a population (Allendorf et al., 2010; Caballero & García-Dorado, 2013; Greenbaum et al., 2014), and it has been suggested that this measure is of key importance in population conservation and management (Foulley & Ollivier, 2006). In this study to evaluate the genetic diversity of the three stocks, heterozygosity, F_{is} and allelic richness were calculated and no correspondence was found between Ho and allelic richness. The Mediterranean stock showed the highest value of H_o and H_e but the lowest value of allelic richness. Interestingly, recent work on Mediterranean swordfish (Righi et al., 2020; Yüncü et al., 2021) found a reduction in mitochondrial haplotypes and haplotype diversity as well as a reduction of females' relative effective population size by investigating the temporal variation of mitochondrial genetic diversity and linked such findings to overfishing. Overfishing has been shown to be one of the most significant causes of ongoing genetic erosion and loss of genetic diversity as demonstrated among a variety of fish species of commercial interest worldwide (Allendorf et al., 2014; Pinsky & Palumbi, 2014).

The application of genome assembly and genome-wide SNPs discovery provided a clear benefit as it allowed better exploration of the impact of allelic richness on protein-coding genes and biological functions. Indeed, these genomic strategies are considered more appropriate than classical neutral markers (e.g., microsatellites) to investigate functional genetic variation and fitness traits in the context of conservation biology (Ouborg et al., 2010). Our chromosome-level assessment of allelic richness showed distinct patterns among geographical areas, suggesting that specific genomic regions related to chromosomes 3,5 and 8 are under differential selection. This points to the need to consider genetic diversity changes on these particular genomic regions to further swordfish stock monitoring. Interestingly, in the rainbow trout *Oncorhynchus mykiss*, Martinez et al., (2011) identified genomic regions containing quantitative trait loci associated with reproduction (i.e., egg development, spawning time) and other life history variations affected by natural selection and genomic adaptation to an environment under human-driven changes. In the present study, when investigating allelic richness of protein-coding genes among the three different stocks, a total of 40 genes were identified between North Atlantic and Mediterranean, 35 genes between South Atlantic and Mediterranean and only 1 gene between North and South Atlantic. Among them, several genes, involved in detoxification pathways, innate immune response, vitamins metabolism and up-take, and neuroendocrine signalling showed greater allelic richness in the Atlantic stocks than in the Mediterranean one. The first gene identified is the aryl hydrocarbon receptor nuclear translocator-like protein 1 (*arntl*). The arnt gene encodes a protein that forms a complex with the aryl hydrocarbon receptor (*ahr*) and it binds to xenobiotic responsive elements in responsive genes upon polycyclic or halogenated aromatic hydrocarbon exposure (Hankinson, 1995). Therefore, it represents a key gene that regulates a variety of enzymes involved in the metabolism of xenobiotics and mediates the toxicity of polycyclic or halogenated aromatic hydrocarbons (Carney et al., 2006; Tanguay et al., 2003), with recent evidence pointing out to the immune system also as a potential target of this gene (Segner et al., 2021). The second gene identified was the complement component 1, q subcomponent-like 3a (*c1ql3a*). The *c1ql3a* gene encodes a q-subcomponent of the complement component 1 and might participate in the fish innate immune response (Ghai et al., 2007). Interestingly, the closely related gene c1ql3b had variants strongly associated with population lifespan in the three-spined stickleback *Gasterosteus aculeatus*, suggesting a link between life history traits and immune variation (Whiting et al., 2018). Noteworthy, in addition to *arntl* and *c1ql3a*, two genes involved in vitamin metabolism showed greater allelic richness in the Atlantic stocks than in the Mediterranean one. The first is the thiamine pyrophosphokinase (tpk1) which is the enzyme responsible for the addition of phosphate groups to thiamine monophosphate, and indeed, is crucial for the synthesis of active form of Thiamine (vitamin B1) (Harder et al., 2018). B1 is an essential vitamin that is required for a range of metabolic functions in fish (Bettendorff et al., 2013). Thiamine deficiency affects all life stages of fish, including sublethal effects and direct mortality, and has emerged as a possible contributor to decreased survival and reduced reproductive success leading to population declines in a variety of fish taxa (Balk et al., 2016; Brown et al., 2005; Ketola et al., 2000). The second gene involved in vitamin metabolism is the endocytic receptor cubilin (cubn) which in fish mediates the intestinal uptake of intrinsic factor–vitamin B12 (Calduch-Giner et al., 2016). A recent study in zebrafish demonstrated that parental vitamin deficiency affects fertility of breeders and affects the embryonic expression of immune-, lipid transport- and apolipoprotein genes (Skjærven et al., 2016). The last gene of interest is the 5-hydroxytryptamine receptor 1 (htr1a), the receptor of the Serotonin (5-hydroxytryptamine, 5-HT) (Winberg & Nilsson, 1993). In fish, central 5-HT is responsive to multiple stressors, for example, social interaction, nutritional status, and immune challenges, controlling in turn agonistic behavior, stress responses and endocrine functions (Winberg & Thörnqvist, 2016). These results indicate that the Mediterranean swordfish stock is losing allelic richness in some functional genes related to immune, detoxification and neuroendocrine systems and vitamin metabolism. This allelic richness loss might therefore compromise the ability of this stock to adapt to a changing environment. Indeed, the fact that mean allelic richness and heterozygosity showed opposite trends in the Mediterranean, seems to be compatible with selective pressures acting on protein-coding genes. These findings, along with its overfished status and ongoing recovery plan (ICCAT, 2016, 2017b; Saber et al., 2020), suggests genetic vulnerability in the Mediterranean stock as also highlighted by previous authors (Righi et al., 2020; Yüncü et al., 2021). Our finding of weak genetic differentiation between North and South Atlantic stocks is notable. To understand the fine population structure, Bayesian clustering analyses were completed and confirmed the results of PCA and p-distance matrices: two clusters related to the two stocks of the Atlantic Ocean were detected at the magnitude K=3, an aspect that could be due to no evident geographic barriers and no prevented admixture between the two Atlantic stocks. The two stocks share the same allelic frequencies but in a different percentage. Noteworthy, a gradient of genotypes was found from North to South Atlantic and might be the result of the reproductive behavior of specimens from the North Atlantic. In the North-Atlantic Ocean, two spawning areas have been previously detected, one in the Sargasso Sea and the other in the Lesser Antilles, while a South Atlantic Ocean spawning area was detected around the Equator (5°N–5°S) (Arocha, 2007; Mejuto & García-Cortés, 2014). Furthermore, tagging studies revealed the horizontal movement of swordfish during fall from the North Atlantic to more equatorial areas (20-30°N) and back to more Northern areas in the spring and summer to feed (Abascal et al., 2015; Braun et al., 2019; Neilson et al., 2014). Our results suggest that the East-Central Atlantic area is a mixing area between North and South Atlantic stocks. Interestingly, no mixing was shown between South Atlantic and Mediterranean stocks but confirming this observation will require additional sampling. The results achieved in this study, in addition to the genetic differentiation characterization of the three swordfish stocks, shed new light in the placement of the boundaries that separate them, supporting and deepening the findings of Smith, 2015. Specifically, the range of the South Atlantic population extends to latitudes higher than the 5°N ICCAT management boundary, mixing with the North Atlantic stock up to latitudes of 25°N. Likewise the Mediterranean population extends beyond the current management boundary at the Strait of Gibraltar, mixing into the East-North Atlantic with the North Atlantic population westwards up to 20°W. Further, admixture zones, characterized by asymmetric contributions of adjacent populations within samples, are confined to the Northeast Atlantic. While South Atlantic and Mediterranean migrants were identified within these Northeast Atlantic admixture zones, no North Atlantic migrants were identified respectively in these two neighbouring basins. Our analysis of 26,324 SNPs suggests that among the swordfish caught in the East-North Atlantic in Portugal and Canary Islands sampling areas, some 10-14% belong to Mediterranean stock; while among those caught in seas adjacent to the Western Sahara (latitudes of 22°N), some 10% belong to the South Atlantic. In this light, the presence of these animals should be considered when developing stock population models and developing management advice on stock status.

3.6 Conclusion

This study deepened the knowledge about the genetic structure and variation among the three swordfish stocks: North Atlantic, South Atlantic and Mediterranean. The Mediterranean stock is strongly genetically differentiated from the two Atlantic stocks. By the contrary, the North Atlantic and the South Atlantic stocks are weakly differentiated, and their differentiation is detectable only with few statistical tests. The coupling of genome-wide SNPs analysis with a genome assembly identified allelic richness as the optimal genetic diversity index to monitor these stocks. Thanks to the allelic richness analysis at genomic level, it was possible to identify for the first time the weaknesses of evolutionary potential of the Mediterranean stock which is losing allelic richness of important genes associated with detoxification, immune response, vitamin up-take and metabolism and serotonin signalling. In addition, this study confirmed that swordfish fisheries management should consider that in the East-North Atlantic is a mixing area for all three stocks and the presence of these animals should be considered when genetic variability is monitored in this area. These results provide new and robust tools useful for a science-based decision-making process for the management of swordfish fisheries in the Atlantic and Mediterranean, as well as a solid basis for improved monitoring not only for the variability and genetic structure but also of the fitness and evolutionary potential of the three stocks.

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CHAPTER 4

Histological investigation on reproductive biology of the Atlantic bluefin tuna (*Thunnus thynnus*) in the Central Adriatic Sea: differences between males and females

(MANUSCRIPT IN PREPARATION)

4.1 Abstract

The Atlantic Bluefin Tuna *Thunnus thynnus* are large and highly migratory fish that support important fisheries. At present, little information is available on the Atlantic bluefin tuna in the Central Adriatic Sea. Therefore, this study focused on the gonadal status of both males and females from February to November in the Central Adriatic Sea. 65 specimens, 33 females and 32 males) were collected in the Central Adriatic Sea between February and November 2021. The gutted weight of each animal was measured, and gonad samples were stored for histological analysis. This study demonstrated that the largest animals (> 65 kg) did not migrate to the Central Adriatic Sea for feeding. In addition, histopathological analysis of the ovaries and testes revealed that only females showed alterations in oocytes, including pre-vitellogenic atresia, nucleic vesicles of atretic oocytes, and interstitial tissue, including necrosis, fibrosis, and infiltrates. Furthermore, for the first time melanomacrophage centers (MMCs) were found in the ovaries and testes of Atlantic Bluefin Tuna. In the ovary, the density and number of MMCs did not change in relation to size range and month. However, the density and number of MMCs changed in males in relation to the months, showing a decrease from February to August, and an increase from August to October. In addition, females showed a higher density and number of MMCs than males did. The present study revealed that the reproductive status of Atlantic bluefin tuna females caught in the Central Adriatic Sea is compromised compared to that of males, and the impairment of oogenesis could result in the correct formation and quality of gametes.

4.2 Introduction

The *Thunnus thynnus*, Atlantic bluefin tuna (ABFT), is the largest tuna, reaching 3.3 m and 725 kg (Cort et al., 2013), and is an important fisheries resource. At present, little information is available regarding the gonadal status of the Atlantic bluefin tuna in the Adriatic Sea (Corriero et al. 2013; Mladineo et al. 2008; Šegvić Bubić et al. 2011; Zupa et al. 2009). The reproductive season in the Mediterranean Sea starts in the Levantine Sea in May (Karakulak et al., 2004) and ends in July in the central and western Mediterranean (Abascal et al., 2004; Corriero et al., 2003; Susca et al., 2001; Zupa et al., 2009), when the water temperatures is 23°C approximately (Schaefer, 2001). During the last few decades, the Mediterranean Sea has been exposed to climate change and pollutants owing to an increase in human activities (Ramos-Miras et al., 2019). Several studies evidenced that pollutants exposure could impair reproduction of fish since the high sensitivity of gonads to chemicals compaound (Spanò et al., 2004; Tillitt et al., 2010), and histopathology approach of gonad has achieved increasing interest to assess reproductive capacity and status of fish affected by a large variety of environmental stressors (Spanò et al., 2004; Tillitt et al., 2010; Abdel-Kader & Mourad, 2019; Elgaml et al., 2019a; Hou et al., 2018; Kaptaner, 2015; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021; Senarat et al., 2017; Singh Brraich & Jangu, 2015; Song et al., 2021; Yadav Rajkiya Mahavidyalaya Unnao et al., 2018). Ovarian and testis histopathological biomarkers, such as pre-vitellogenic atresia, necrosis, the presence of infiltrates, intersex (the presence of oocytes in the testis tissue), and vacuolation of spermatocytes and spermatids, were associated with exposure to several pollutants (Rzepkowska et al., 2020; Bhaisare et al., 2022; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021). An additional biomarker of pathological conditions in fish ovaries and testes is the massive occurrence of melanomacrophage centers (MMCs) (Ardeshir et al., 2022; Das Neves et al., 2018; Doherty et al., 2019; Puttipong et al., 2022; Silva et al., 2022). These centers consist of pigmented phagocytic cells, and are considered simple measures of innate and adaptive immunity in fish (Ardeshir et al., 2022; Blazer, 2002; Doherty et al., 2019). MMCs differ in shape, size, pigment content, and distribution in organs (e.g., the liver, spleen, kidney, and gonads) and play complex physiological and immunological roles (Sayed & Younes, 2017). These aggregated cells contain three types of pigments: melanin, hemosiderin, and lipofuscin. Hemosiderin is found during the degradation and phagocytosis of erythrocytes, followed by iron, which is carried out by melanomacrophages (Agius and Roberts 2003). Lipofuscin is found in MMCs during the beta-oxidation of fatty acids due to pollutant exposure or aging in animals (Imai et al., 2022). Melanin (Kalita et al., 2019) is also involved in the neutralization of the free radicals released during cell membrane rupture (Qualhato et al., 2018). Previous studies have shown that the morphology and pigment content of MMCs respond to a variety of internal and external factors such as temperature, age, sex, hypoxia, hibernation, xenobiotics, season, infection, and hormones (Mela et al., 2007; Steinel & Bolnick, 2017; Zieri et al., 2015). This study aimed to evaluate the gonadal status of *male* *and female Thunnus thynnus* caught in the central Adriatic Sea between February and November 2021. With this goal, histopathological changes in the ovaries were identified, whereas no alterations were observed in the males. In addition, the density and number of MMCs in the ovaries and testes were quantified and found to be related to the month and size. Using a histological approach, we highlighted the compromised reproductive status of Atlantic bluefin tuna females caught in the Central Adriatic Sea compared with that of males.

4.3 Materials and Methods

Sampling activities

The gutted weight (GW) of landed specimens (N= 568) caught by a long liner from February to November 2021 in the central Adriatic Sea was recorded (min=18 kg; max=104.4 kg). Sex was determined macroscopically from 199 specimens (min= 19.1 kg; max=104.4 kg) to assess the sex-frequency distribution. Ovaries and testes were collected from 65 specimens (33 females and 32 males), with a range of 21 kg to 104.4 kg of GW. The samples were collected according to the guidelines of the International Commission for the Conservation of the Atlantic Tuna (ICCAT). The procedures did not include animal experimentation and ethical approval was not necessary according to Italian legislation (D.L. 4 of March 2014, n. 26, art. 2).

Histological analysis

Samples of ovaries and testes (~3 cm3) were fixed in formaldehyde-glutaraldehyde solution (NaH2PO4·H2O+NaOH+formaldehyde 36.5%+glutaraldehyde 25%+H2O) and stored at 4 °C until histological analysis. Ovary and testis samples were serially dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Sections (5 µm) were cut using a microtome (model RM2125 RTS; Leica Biosystems, Wetzlar, Germany), stained with Mayer's hematoxylin and eosin, and examined under a microscope (Axio Imager 2; Zeiss, Oberkochen, Germany). Hematoxylin and eosin (H&E) was considered a standard staining method to identify melanin, Perl's Prussian Blue staining for hemosiderin and Sudan Black B and autofluorescence (excitation 596 nm, emission 600-610 nm) for lipofuscin (Dang et al., 2019). The analysis of ovarian alterations was performed in six sections, with a separation of 50 µm between each section, taken with a 10x objective. Alterations were identified according to the methods described by Nibamureke and Wagenaar (2020) and Rodrigues et al. (2021). The alterations of ovaries were divided into alterations of oocytes, such as pre-vitellogenic atresia and nucleic vesicles of atretic oocytes, and alterations of interstitial tissue, such as necrosis, fibrosis, and infiltration of white blood cells. Fibrosis was determined macroscopically, whereas other alterations were histologically determined. Alterations in the testes were divided into alterations in male reproductive cells, such as vacuolation and nuclear alterations of spermatocytes, vacuolation and intersex of spermatids, vacuolation of spermatogonia, and interstitial tissue, such as vacuolation, necrosis, and infiltration of white blood cells. Histological alterations were considered to be present in an animal only if present in four out of six sections, and subsequently, the percentage of females and males affected by single alterations and in relation to months and size range were evaluated. The animals were considered healthy only in the absence of alterations. Quantification of MMCs was performed in the same sections of ovaries and testes obtained with a 20x objective (digital field area = 149,838 µm^2) (Imai et al., 2022). The separation between sections was 50 µm. The density of MMCs and their number per mm2 of gonadal parenchyma were measured using Fiji software (Schindelin et al., 2012). The density of the MMCs was expressed as µm^2/mm^2.

Statistical analysis

Data from MMCs were first checked for normality using the Shapiro test. Pearson’s correlation test was performed for the density and number of MMCs and GW (kg) in the R-studio environment using the cor.test command. The density and number of MMCs were analyzed in relation to the size range and months by ANOVA using GraphPad Prism 6 version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The density and number of MMCs in females and males were compared by t-test using GraphPad Prism 6 version 6.00 for Windows (GraphPad Software, La Jolla California USA, https://www.graphpad.com/).

4.4 Results

Swordfish size-frequency distributions

The overall and per-month size distributions of the landed swordfish are shown in Figures 1 and 2, respectively. The overall mean swordfish size was 35,6 kg (GW), ranging from 18 to 104.4 kg GW. The most represented size-ranges were 25-35 kg and 35-45 kg (Fig.1).

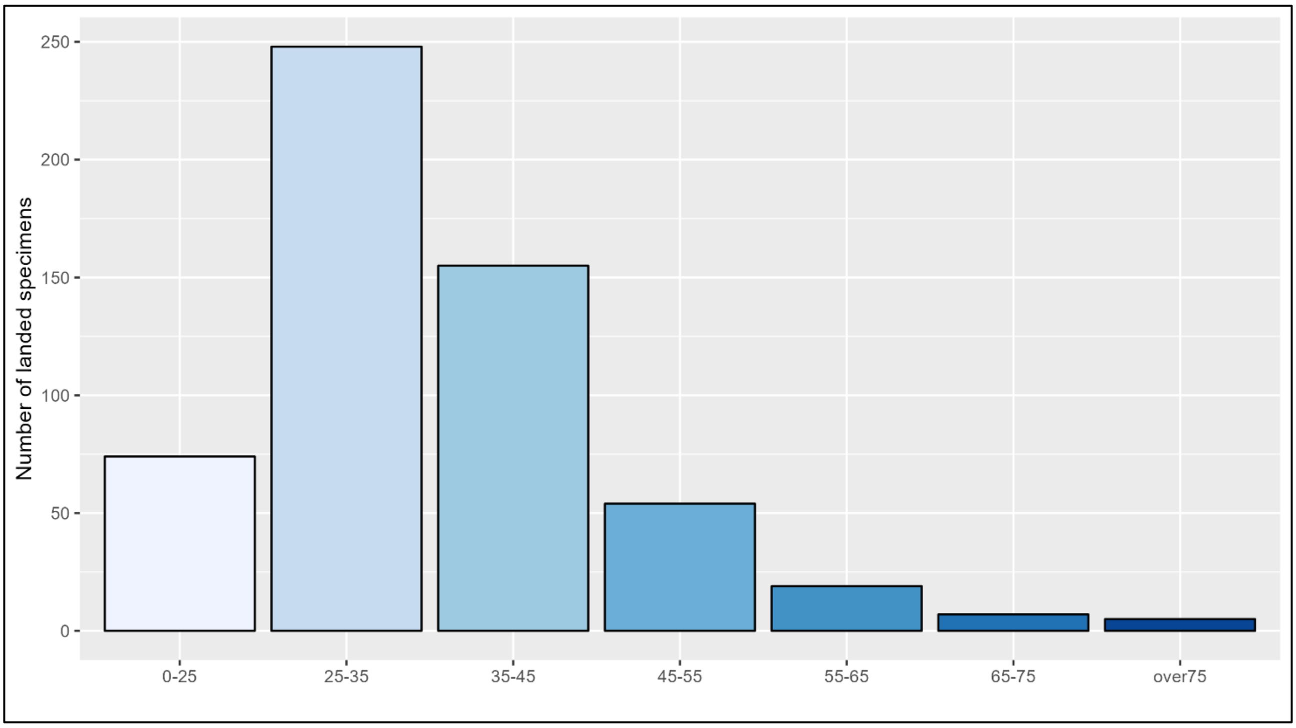

Figure 1 - Total size-frequency distribution. The colour of the bar plot represents the size range.

The seasonal size-frequency distribution showed that the most represented size range was 25–35 kg in August and September (Fig. 2).

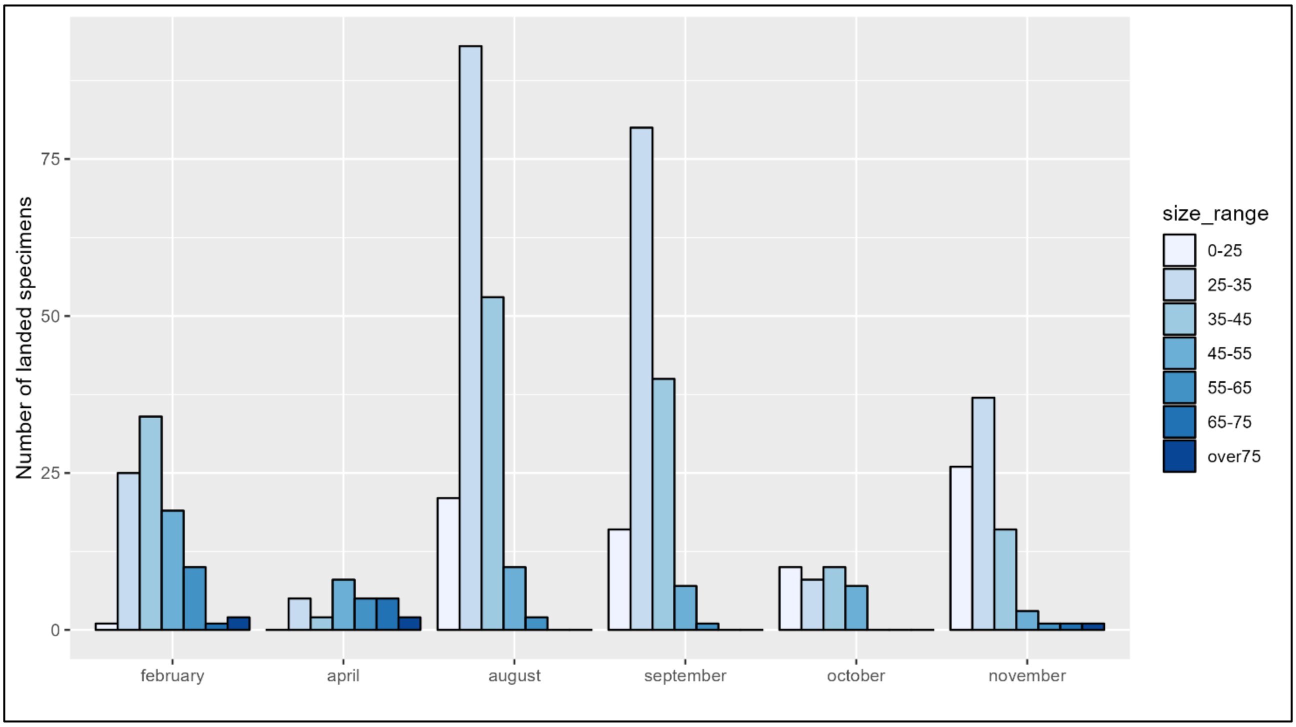

Figure 2 - Seasonal size-frequency distribution. The color of the bar plot represents the size range.

A total of 108 females and 91 males were included in the study. Most females landed in September and November, whereas most males landed in August and November (Fig.3).

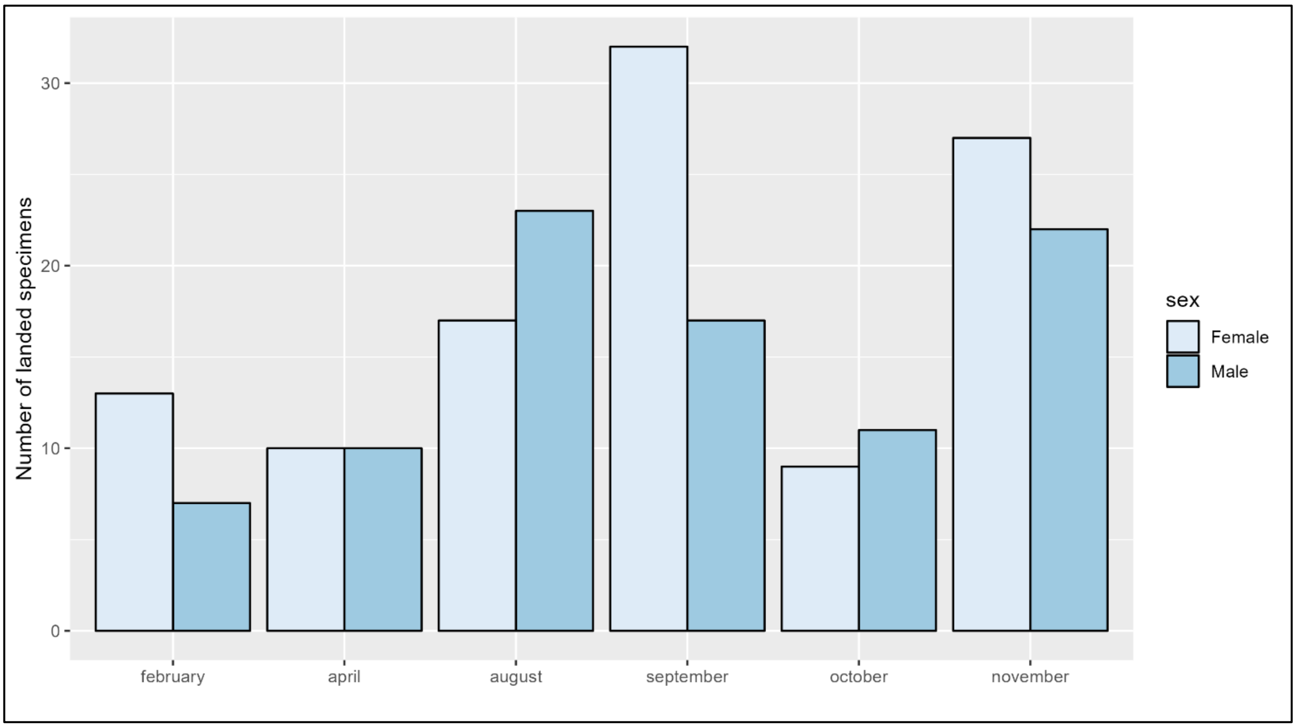

Figure 3 - Total sex–frequency distribution.

Histopathological analysis of ovaries and testis

All ovaries were classified as resting (R), with oogonia and primary oocytes with circumnuclear oil droplets, whereas all testes were in the spent (S) phase (Fig. 4).

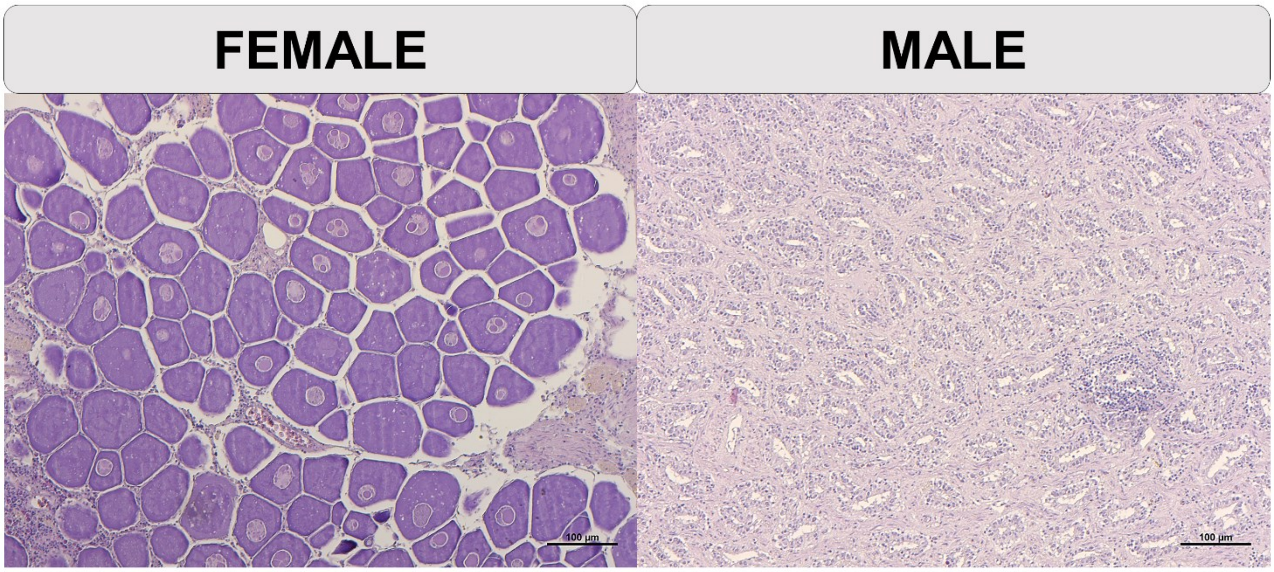

Figure 4 - Photomicrographs of the ovary and testis. Bar = 100µm

Five alterations were identified in the ovaries: two alterations in oocytes (atresia of pre-vitellogenic oocytes and nucleic vesicles of atretic oocytes) and 3 of interstitial tissues (necrosis, infiltrates of white blood cells, and fibrosis) (Fig. 5).

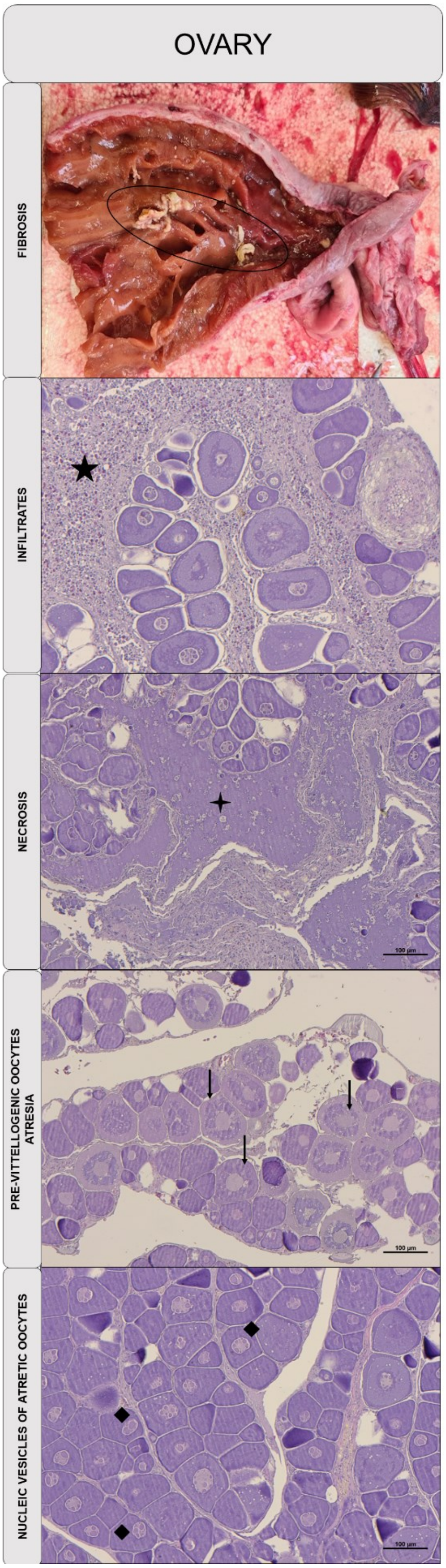

Figure 5 - Immature and mature ovaries: Fibrosis (black circle); Infiltrates of white blood cells (black star); Necrosis (black four points star); Pre-vitellogenic oocytes atresia (bold arrow) and nucleic vesicles of atretic oocytes (black rhombus)

None of the females were healthy, while fibrosis and infiltrates of white blood cells were the most frequent alterations, as well as atresia of pre-vitellogenic oocytes (Fig. 6A). Investigating the occurrence of ovarian alterations in relation to months, it was evident that the nucleic vesicles of atretic oocytes were found only in February, April, August, and September, while most of the necrosis was found in November (Fig. 6B). Investigating the occurrence of ovarian alterations in relation to the size of females, it was evident that the alteration of interstitial tissue was found in all size ranges, except over 75 kg. Nucleic vesicles of atretic oocytes and atresia of pre-vitellogenic oocytes were found in all size ranges. (Fig. 6C). No alterations were recorded in the male gonads, which were healthy.

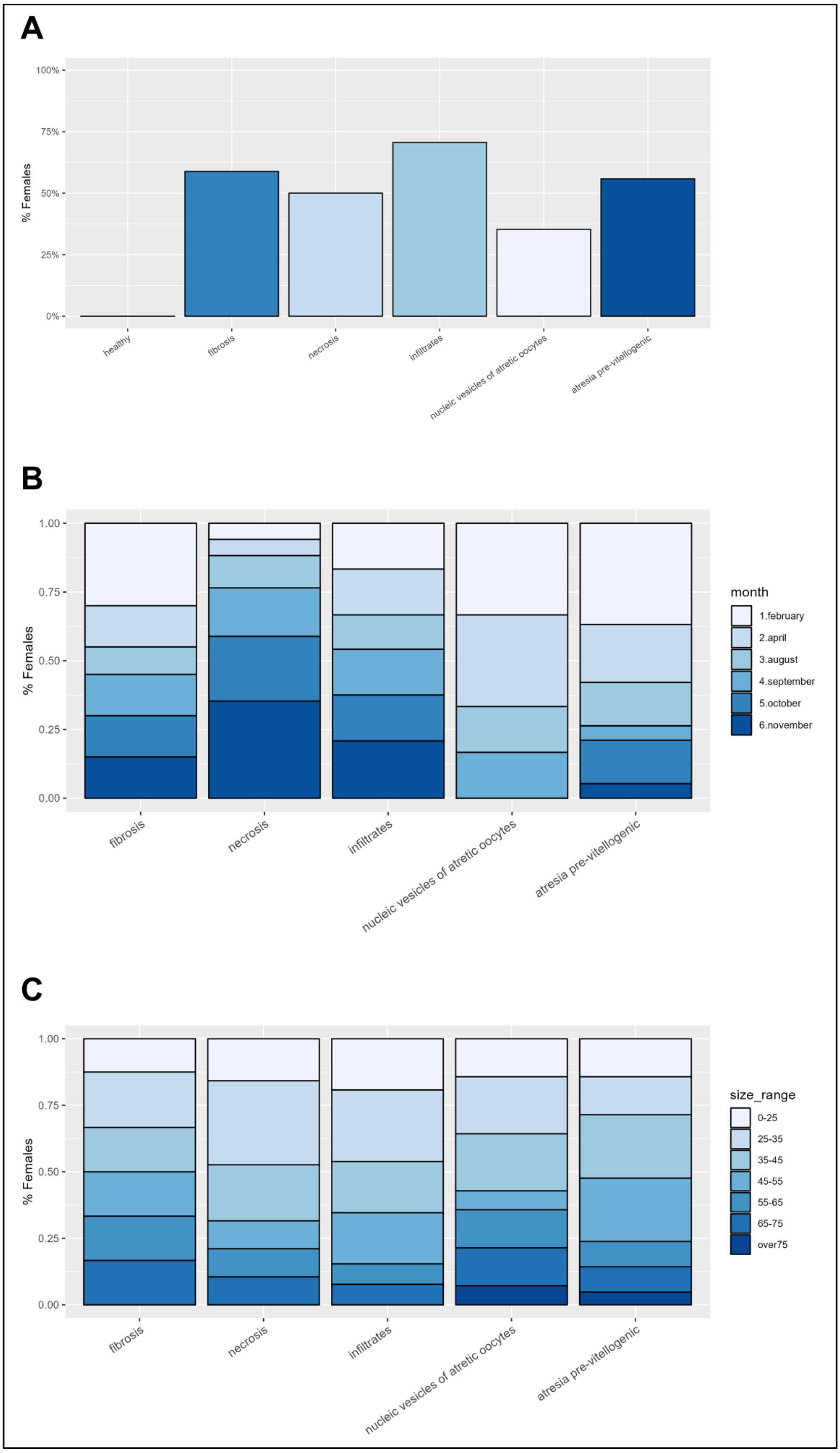

Figure 6 - Frequencies of alterations in females: A) total frequencies of alterations; B) related to month and C) related to size range.

MMCs quantification related to weight

The density and the number of MMCs in all samples were correlated to fish weight (GW). No significant positive correlation was observed between fish weight and either density or number of MMCs (p-value >0.05, Pearson's correlation= 0.16 and 0.25, dMMCs and MMCs/mm2, respectively) (Table 1).

	Weight	**dMMCs**	**MMCs/mm^2**
Weight	**NA**	0.164	0.252
dMMCs	0.164	**NA**	**0.678**
MMCs/mm^2	0.252	**0.678**	**NA**

Table 1 - Pearson correlations between MMCs density (μm^2/mm^2) and number per mm^2 and fish size (GW or kg). Significant correlations (P-VALUE < 0.05) are indicated in bold.

Similar results were obtained by correlating the density and number of MMCs of females and males, respectively, to fish weight (GW) (p-value >0.05, Pearson's correlation= 0.28 and 0.28, dMMCs and MMCs/mm2, respectively, in females; p-value >0.05, Pearson's correlation= 0.19 and 0.26, dMMCs and MMCs/mm2, respectively, in males) (Table 2).

		Male	
	Weight	**dMMCs**	**MMCs/mm^2**
Weight	**NA**	0.195	0.264
dMMCs	0.195	**NA**	**0.627**
MMCs/mm^2	0.264	**0.627**	**NA**
		Female	
	Weight	**dMMCs**	**MMCs/mm^2**
Weight	**NA**	0.287	0.28
dMMCs	0.287	**NA**	**0.651**
MMCs/mm^2	0.28	**0.651**	**NA**

Table 2 - Pearson correlations between MMCs density (μm^2/mm^2) and number per mm^2 and fish size (GW or kg) of males and females Significant correlations (P-VALUE < 0.05) are indicated in bold.

MMCs shapes

MMCs displayed different shapes (round/oval or polymorphic) and sizes in the ovaries and testes. In fact, males showed round/oval shapes and a limited variation in size, and the three pigments, characteristic of MMCs, were found. Furthermore, lipofuscin autofluorescence detected more MMCs than the other staining methods. Females also showed a polymorphic shape and a variation in size, for example, small centers or closely aggregated that could form large areas of MMCs. In addition, MMCs showed three pigments similar to males (Fig.7).

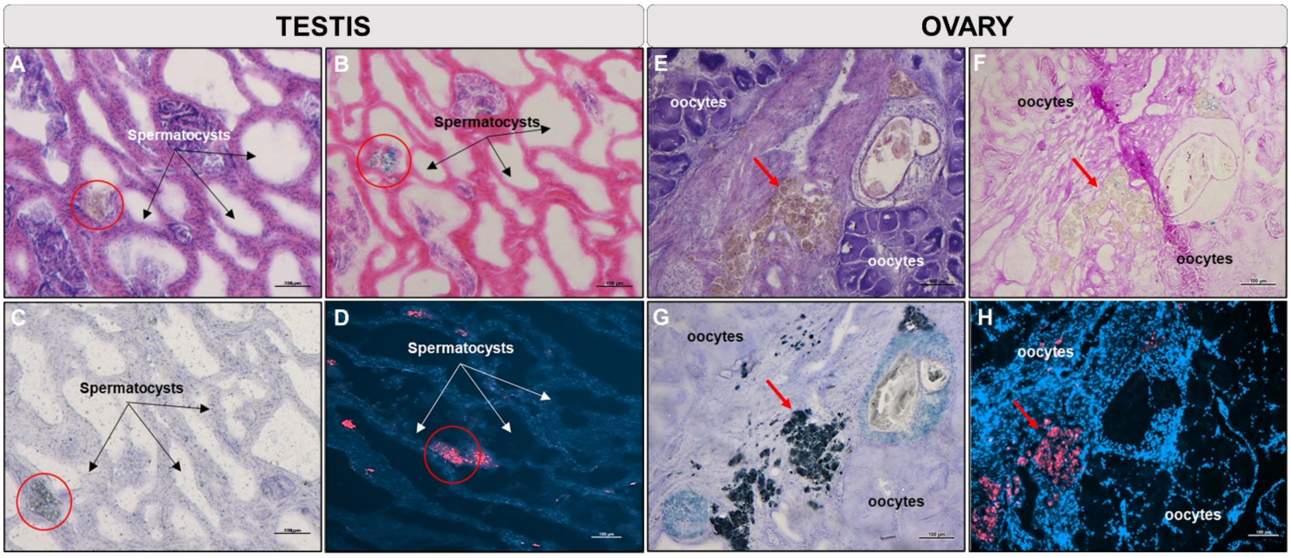

Figure 7 - Characterization of MMCs in ovaries and testes. A-D) MMCs in the testis were round/oval in shape and small in size, with melanin (A), hemosiderin (B), and lipofuscin (C-D). E-H) MMCs in the ovary showed polymorphic shapes and large sizes, with melanin (E), hemosiderin (F), and lipofuscin (G-H).

MMCs quantification related to sex, size-range and month

MMC was independently associated with sex, size range, and month. No significant differences were found in the density and number of MMCs related to the size range in males and females (Fig. 8).

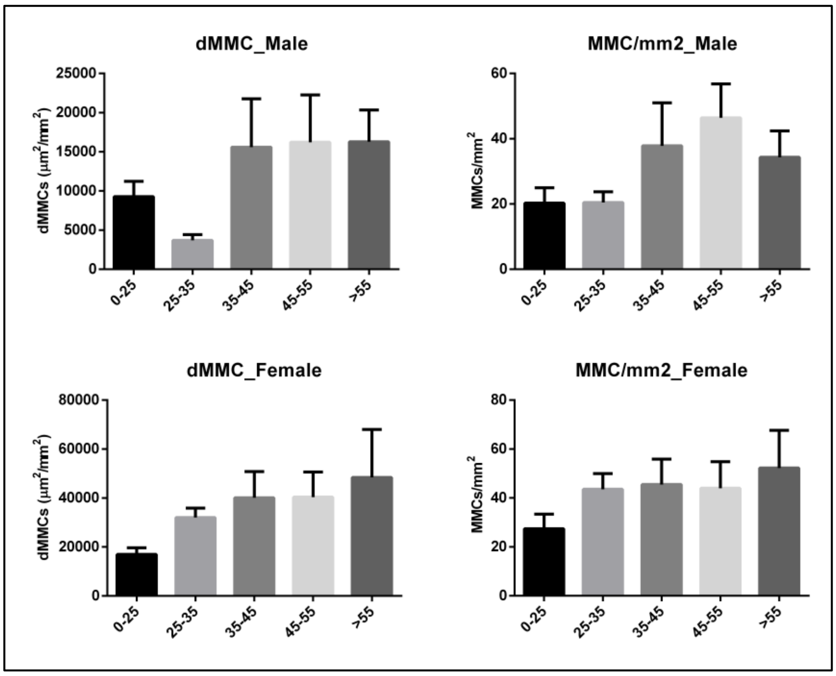

Figure 8 - Density and number per mm^2 of melanomacrophage centers (MMCs) of males and females are related to size range.

A decreasing trend in the density of MMCs was found (p-value <0.05) from February to August, and an increasing trend from August to October was found in males (p-value < 0.05), while no significant differences were found in females (Fig. 9).

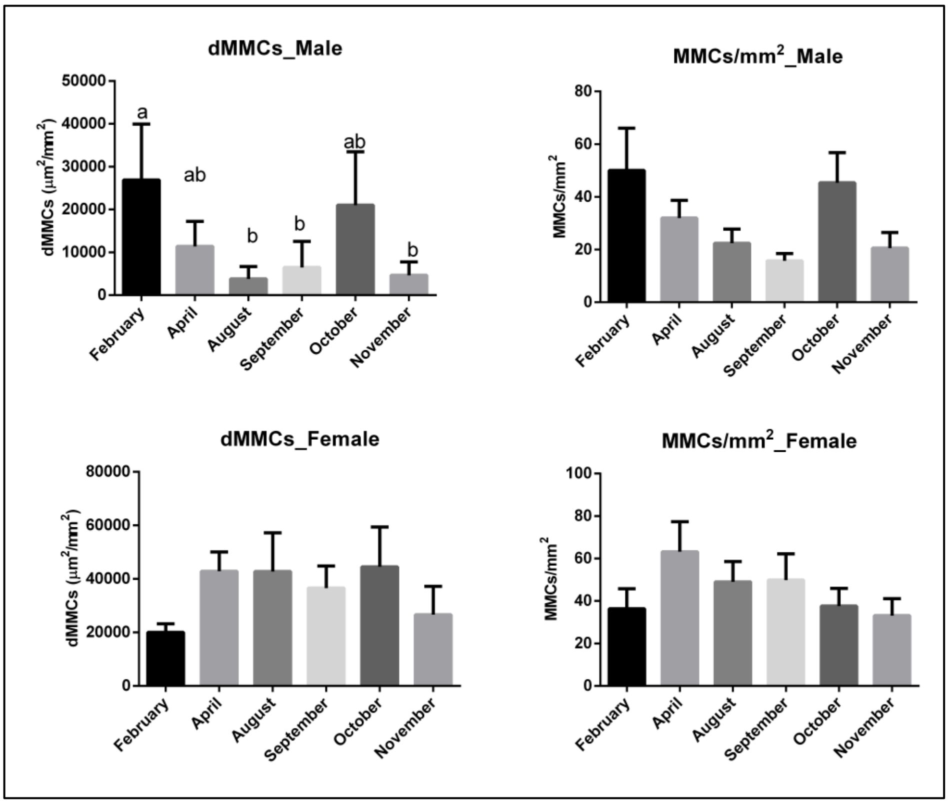

Figure 9 - Density and number per mm^2 of melanomacrophage centers (MMCs) of males and females related to month. Different letters indicate significant difference.

The density and number of MMCs were also investigated in relation to the sex per month. The density of MMCs showed a significant increase in females caught in April, August, and September compared to males (p-value <0.05), whereas no significant differences were found between females and males caught in February, October, and November. The number of MMCs per mm2 was significantly higher in females caught in August and September than in males (p-value <0.05) (Fig. 10).

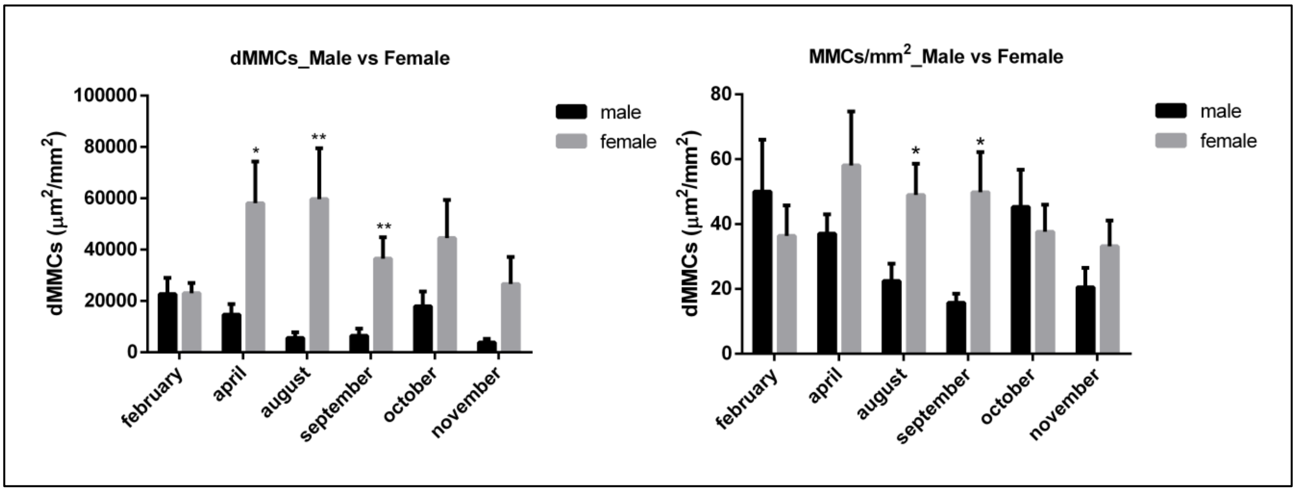

Figure 10 - Density and number per mm^2 of melanomacrophage centers (MMCs) related to sex/month. * Indicate a significant difference. (*=$p<0.05$, **=$p<0.01$).

4.5 Discussion

The main pelagic fish commercially exploited in the Mediterranean Sea is the Atlantic bluefin tuna, *Thunnus thynnus* (ICCAT,2020). Little information is available regarding this species in the Adriatic Sea (Corriero et al., 2013; Mladineo et al., 2008; Šegvić Bubić et al., 2011; Zupa et al., 2009). In particular, the gonadal status of male and female T. *thynnus* caught in the Central Adriatic Sea remains unknown. The landings monitored in this study showed that the most represented size ranges were under 45 kg in the Central Adriatic Sea. The class of over 65 kg appears to be the least fished class, suggesting that the largest animals do not migrate in the Central Adriatic Sea during the months monitored in this study. Histological analysis revealed that all females were in the spent phase, independent of size. However, females under 35 kg seemed to be the first to finish spawning because they were the first to enter the Adriatic (in August and September) for foraging with the gonad. These results are in agreement with the electronic tagging performed by Cermeño et al. (2015), who revealed that Atlantic bluefin tuna migrate in the Adriatic Sea from May to September. Interestingly, some tuna left the Adriatic Sea towards the end of May to reproduce in Sicily and returned to the Adriatic Sea around mid-August. Additionally, five types of alterations in the ovaries were found: three in interstitial tissue and two in oocytes. Alterations in the ovary (vacuolation and atresia of pre-vitellogenin oocytes, necrosis, fibrosis, and infiltrates of white blood cells) were found in all females, and none of the females were found to be healthy. A large proportion of females showed fibrosis and necrosis. The presence of fibrosis and other alterations in the interstitial tissue observed in this study could be related to exposure to chemical pollutants (especially ECDs), as previously demonstrated in fish such as *Danio rerio* (Luzio et al., 2016; Weber et al., 2003), *Dicentrarchus labrax* (Blázquez et al., 1998), *Oryzias latipes* (Kiparissis et al., 2003), *Chalcalburnus tarichi* (Kaptaner & Ünal, 2011), *and Oreochromis niloticus* (Nibamureke & Wagenaar, 2020). In addition, the females showed the same rate of alterations in all months, except for the nucleic vesicles of early atretic perinucleolar oocytes, which were absent in October and November. Alterations in the ovary, both in interstitial tissue and germ cells, may damage the process of gametogenesis and may affect the number of gametes produced, which reduces the reproductive capacity of individuals over the long term (Delbes et al., 2022). The effects of pollutants on ovary and testis have been investigated in previous studies (Abdel-Kader & Mourad, 2019; Elgaml et al., 2019a; Hou et al., 2018; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021; Senarat et al., 2017; Singh Brraich & Jangu, 2015; Song et al., 2021; Yadav Rajkiya Mahavidyalaya Unnao et al., 2018; Ardeshir et al., 2022). In particular, Nibamureke and Wagenaar (2020) investigated the effects of the ARV drug nevirapine on the testes and ovaries of *Oreochromis mossambicus*. They found alterations in oocytes (oocyte atresia and vacuolation of oogonial nests) and interstitial tissues in the ovary and vacuolation of spermatocytes and spermatids in the testes, suggesting that chronic exposure to pollutants, antibiotics, or other chemical compounds may affect both males and females.

Interestingly, in our study, males did not seem to be affected by environmental stressors. Conversely, BFT females are more susceptible to chemical and other pollutants throughout the year, as demonstrated by the lack of monthly differences. In addition, for the first time, MMCs are evidenced in ovaries and testis of ABFT. In other fish species, MMCs have been found in the ovaries and testes, such as *Pangasianodon hypophthalmus* (Senarat et al., 2022) and *Alburnus tarichi* (Kaptaner, 2015). In these two species, the presence of MMCs in the ovaries and testes may be due to exposure to pesticides, ECDs, and heavy metals. Senarat et al., 2022, also, have found MMCs localized nearly impaired oocytes (especially atretic vitellogenic and pre-vitellogenic oocytes), suggesting a strong association between the presence of MMCs and altered oocyte. In our study, the MMCs were not associated with oocytes but were localized in the interstitial tissue, especially in the presence of white blood cell infiltrates. This result is in agreement with Ravaglia and Maggese (1995), who also suggested that MMCs in *Synbranchus marmoratus* could play a role in the degeneration of oocytes and sperm, resorption of non-specific tissue, and inflammatory processes. Furthermore, in this study, the MMCs of the female and male Atlantic blue fin tuna showed different shapes. The females showed a polymorphic shape and a higher presence of lipofuscin, other than a larger size. Therefore, males showed a round/oval shape and a higher presence of hemosiderin, other than a smaller size. The presence of lipofuscin in MMCs of females suggests that they are affected by pollutants that cause the peroxidation of lipids in oocytes phagocytized by MMCs. In males, the presence of hemosiderin may be due to the retention of iron in MMCs (probably after phagocytosis of erythrocytes) as a protective mechanism (Agius & Roberts, 2003; Blazer, 2002). In addition, in our study, females showed a higher density and number of mm2 of MMCs than males. No differences were found in the density and number of MMCs in females related to size range and month, confirming that females are constantly exposed to pollutants. In fact, chronic exposure to pollutants can cause irreversible damage to ovaries, altering functional and physiological processes (Luzio et al., 2016; Nibamureke & Wagenaar, 2020; Rodrigues et al., 2021). In males, a decreasing trend in the density of MMCs was found from February to August and an increasing trend from August to October related to the month. In the case of males that are not affected by alterations, MMCs seem to play a role in the reabsorption of gametes after the reproductive season, as suggested by the increase in MMCs density in the non-reproductive months. In fact, it has been suggested that both follicular cells and immune cells, including macrophages from the blood, act synergistically in gamete resorption (Besseau & Faliex, 1994). In our study, only females were affected by alterations and showed a higher density of MMCs, suggesting that females are more susceptible to environmental stressors throughout the year than males.

4.6 Conclusion

In conclusion, our study highlights for the first time that the reproductive status of Atlantic bluefin tuna females caught in the Central Adriatic Sea is compromised compared with that of males. At the ovarian level, oocyte and interstitial tissue alterations occur, compromising the correct formation and quality of gametes. In addition, MMCs were observed in the ovaries and testes of Thunnus thynnus for the first time. Females showed a higher density and number of MMCs than males. The results of this study suggest that females are more affected by environmental stress throughout the year. In fact, in all sampling months and size ranges, a similar frequency of alterations and density/number of MMCs were observed. It is therefore recommended that further studies be carried out on the liver of females and males to understand whether their overall health status is also impaired.

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CONCLUSIONS

The evaluation of health status and reproductive dynamics underlies the conservation biology of two important fisheries resources, *Xiphias gladius* and *Thunnus thynnus*. In this PhD project, multiple approaches were applied to better understand and to shed light on the health and reproductive status of these two species in Mediterranean Sea.

Chapter 1: A window of vulnerability: chronic environmental stress does not impair reproduction in the swordfish *Xiphias gladius*

1. A transcriptomic approach at hepatic level, shed light on molecular dynamics changes of metabolism, reproduction, immune system, and stress response occurring between immature and mature females during the reproductive season.
2. Mature females invest most of their energy in reproduction instead of the immune system and detoxification compared to immature females, a finding corroborated by both histological and transcriptomic analyses. In fact, mature females show a lower density of lipids in the liver and downregulation of genes involved in lipid biosynthesis than immature females.
3. The competition between AhR and ERs for the same cofactor (arnt2) inhibits the cytochrome p450 signaling pathway and modulates ERs gene expression, which makes mature females more susceptible to environmental stressors than immature females during the reproductive season.
4. Melanomacrophages are identified for the first time in the livers of swordfish females, and the density and number of single (MMs) and centers of melanomacrophages (MMCs) can be used as biomarkers of stress and aging at the hepatic level. In fact, a significant increase in the density of MMCs is found in mature females, in addition to downregulation of the immune system and detoxification-related genes.

Chapter 2: Oogenesis impairment in Swordfish (*Xiphias gladius*) caught in Central Adriatic Sea: differences between mature and immature females

1. This study evidenced that the most landed size-ranges are those at which the females are still immature.
2. Histopathological examination of the ovaries revealed five types of alterations, three in interstitial tissue, and two in oocytes. Pollutants can induce this alteration. In particular, pollutant exposure could reduce the fertility of mature females and cause an incorrect development of ovaries and a delay in acquiring sexual maturation of immature females.
3. MMCs and MMs are found in the liver of swordfish. The assessment of density and number of MMCs and MMs revealed that mature females showed higher values of density and number per mm2 of MMCs and MMs, compared to immature females. This difference could be due to the response to pollutants that affects both reproduction and overall health, as previously identified in other fish species. This result suggests that mature females are more susceptible to environmental stressors than are immature females.
4. Furthermore, the reduced availability of lipids as an energy source in mature females may explain the lower response to environmental stress during the reproductive and non-reproductive seasons.
5. Further studies on males during the reproductive and non-reproductive seasons could add new information on the health status of Mediterranean swordfish to assess whether the health and gonadal status identified in the females persists in the male specimens.

Chapter 3: ddRAD genotyping reveals new insight into the genetic structure and diversity, fitness and evolutionary potential of Atlantic and Mediterranean swordfish stocks

1. In this study, a genome-wide SNPs analysis combined with genome assembly was applied for the first time to better describe the genetic structure of swordfish stocks from the Atlantic Ocean and Mediterranean Sea and to explore their genomic landscape.
2. The Mediterranean stock is strongly genetically differentiated from the two Atlantic stocks. In contrast, the North Atlantic and South Atlantic stocks are weakly differentiated, and their differentiation is detectable only with few statistical tests. Therefore, this strong differentiation should be considered for the separate management of stocks.
3. The Mediterranean population extends beyond the current management boundary at the Strait of Gibraltar, mixing into the East-North Atlantic with the North Atlantic population westward up to 20°W. Furthermore, admixture zones, characterized by asymmetric contributions of adjacent populations within the samples, are confined to the Northeast Atlantic. While South Atlantic and Mediterranean migrants were identified within these Northeast Atlantic admixture zones, no North Atlantic migrants were identified in these two neighboring basins. Therefore, the presence of these animals should be considered when developing stock population models and management advice on stock status.
4. A gradient of genotypes was found from the North to the South Atlantic and might be the result of the reproductive behavior of specimens from the North Atlantic. Our results suggest that the East-Central Atlantic area is a mixed area between North and South Atlantic stocks.
5. The coupling of genome-wide SNPs analysis with genome assembly identified allelic richness as the optimal genetic diversity index to monitor these stocks. Thanks to the allelic richness analysis at the genomic level, it was possible to identify for the first time the weaknesses of the evolutionary potential of the Mediterranean stock, which is the loss of allelic richness of important genes associated with detoxification, immune response, vitamin uptake, metabolism, and serotonin signalling.

Chapter 4: Histological investigation on reproductive biology of the Atlantic bluefin tuna (*Thunnus thynnus*) in the Central Adriatic Sea: differences between males and females

1. This study demonstrated that most landed size ranges are under 45 kg in the Central Adriatic Sea. The histological approach reveals that all females are in spent phase, independently by size. However, the females under 35kg seem to be the first to finish spawning because they are the first to enter the Adriatic (in August and September) for foraging with the gonad totally spent.
2. Five types of alterations in the ovaries were found: three in interstitial tissue and two in oocytes. Alterations in the ovaries (vacuolation and atresia of pre-vitellogenin oocytes, necrosis, fibrosis, and infiltration of white blood cells) were found in all females. In fact, no females were healthy. Most females showed fibrosis and necrosis. Alterations in the ovary, both in interstitial tissue and germ cells, probably caused by pollutants, may damage the process of gametogenesis and may affect the number of gametes produced, which reduces the reproductive capacity of individuals over the long term.
3. In our study, males did not seem to be affected by environmental stressors. Conversely, females are more susceptible to chemical and other pollutants throughout the year, as demonstrated by no differences related to month, compared to males.
4. For the first time, MMCs are evidenced in ovaries and testis of Atlantic Bluefin Tuna. MMCs are not associated with oocytes, but are localized in the interstitial tissue, especially in the presence of white blood cell infiltrates.
5. In this study, the MMCs of female and male Atlantic blue fin tuna showed different shapes. The females showed a polymorphic shape and a higher presence of lipofuscin, other than a larger size. Therefore, the males showed a round/oval shape and a higher presence of hemosiderin, other than a smaller size. Probably, the presence of lipofuscin in MMCs of females suggest that they are affected by pollutants that cause the peroxidation of lipids of oocytes, phagocytized by MMCs. In males, the presence of hemosiderin could be due to the retention of iron in MMCs (probably after the phagocytosis of erythrocytes) as a protective mechanism.
6. No differences are found in density and number of MMCs in female related to size-range and months, confirming that females are constantly exposed to pollutants. Chronic exposure to pollutants can cause irreversible damage to the ovaries, altering functional and physiological processes. In males, a decreasing trend of density of MMCs were found from February to August and an increase trend from August to October related to month. In the case of males that are not affected by alterations, MMCs seem to play a role in the reabsorption of gametes after the reproductive season, as suggested by the increase in MMCs density during non-reproductive months.

Concluding remarks

The evaluation of the health status and reproductive dynamics underlies the conservation biology of *Xiphias gladius* and *Thunnus thynnus* in the Mediterranean Sea. In fisheries management, the application of multidisciplinary approaches (e.g., histological analysis, genomic, and transcriptomic approaches) has proven to be an effective solution to determine the genetic structure of populations and shed light on the health and reproductive status of these two species, supporting a science-based decision-making process in the context of fishery management of stocks.