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REGIONE AUTÒNOMA DE SARDIGNA
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FSE 2007-2013
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obiettivo competitività regionale e occupazione

UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO IN

SCIENZE VETERINARIE

INDIRIZZO: **Patologia e Clinica Animale (XXVIII CICLO)**

Histopathological and immunohistochemical characterization of host-parasite interaction in visceral organs of mullets (Osteichthyes: Mugilidae) from Sardinian lagoons

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La presente tesi è stata prodotta nell'ambito della Scuola di Dottorato in "Scienze Veterinarie" dell'Università degli Studi di Sassari, a.a. 2014/2015 – XXVIII ciclo, con il supporto di una borsa di studio finanziata con le risorse del P.O.R. SARDEGNA F.S.E. 2007-2013 - Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l.3.1.

Abstract

Parasitic diseases in fish constitute a limiting factor for wild fishes and for aquaculture production. Extensive aquaculture in Sardinia is an important economical and traditional issue and mullets represent the most important product of this farming system. The aim of this study was to evaluate host-parasite interaction in visceral organs of Mugilidae species from Sardinian lagoons. A total of 239 mullets of different species were collected from four Sardinian lagoons. Lesions were examined macroscopically and histologically and also using histochemical and immunohistochemical techniques. Prevalence of Metazoan parasites (Digenean metacercariae, *Myxobolus* spp. and *Polysporoplasma mugilis*) were evaluated. Metacercariae and *Myxobolus* spp infections showed a similar prevalence in all lagoons, whereas a hight prevalence of *P.mugilis* was observed in Cabras lagoon. Kidney was the most affected organ both for Metacercarie e *Myxobolus* species. Cellular immune response displayed a particularly relevant role with the development of chronic granulomatous reaction against parasites. Macrophages, epithelioid cells and fibroblasts were identified by immunohistochemestry as the main constitutive cell populations of granulomas but also eosinophilic granular cells and rodlet cells were associated to this response. Study of the granuloma structures revealed that Mugilidae displayed a common response against different metazoan parasites in visceral organs.

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1. Aquaculture and main parasitic disease of Teleosts

1.1. Definition of aquaculture

Aquaculture is the farming of aquatic organisms (fish, mollusks, crustaceans, aquatic plants, turtles and amphibians) in which human intervention is directed to increase production by providing feeding, protection from predators and stocking (FAO, 1990-2015).

1.1.1. Main aquaculture systems

Aquaculture made its first appearance when men started to manage natural resources, with the entrapment of wild fishes in lagoons, small lakes or ponds, in order to benefit from an increased availability of food (EU Commission- Aquaculture, 2015). First signaling of aquaculture is dated in the Neolithic age in Europe (4000 B.C.) and in the fifth century B.C., carp represented the most advanced example of aquaculture in China. During centuries humans developed different techniques and management systems to bred aquatic species (fish, crustaceans and mollusks) to obtain the best from marine resources, coastal and inland environments.

In Middle Age these techniques developed mainly in monasteries where, in order to supply protein intake and substitute meat during religious fasting periods, fishes were reared in ponds. During the same period in southern Europe ponds and lagoons have been exploited to confine fish after the tide, such as seabasses, seabreams and mullets. These forms of aquaculture are still present in Europe and are considered traditional extensive fish farming practices. (EU Commission- Aquaculture, 2015).

In the last decades intensive aquaculture has become the first producer of marine farmed species, improving economic condition in many areas of European community (Piccioli A., 2001; Anras L. et al., 2010).

Although these progress, aquaculture provide only 10% of total seafood consumption, and the 65% is imported from other world areas, proving that European aquaculture is still not able to

fill the gap between consumer request and production. Filling this gap will improve economic sustainability of aquaculture production by creating between 3000 and 4000 full time-jobs for each percentage point of EU products consumed. European community has gathered this need and put it inside the Europe 2020 strategy, developing a dedicated program, named Blue Growth Strategy, to support aquiculture improvement and seafood quality (European Commission, COM (2013) 229 final).

However, expanding aquaculture is considered to be a possible threat for environmental impact, due to farm discharges or waste products on flora and fauna and for the breeding of newly introduced species. European community has already adopted a plan to ensure biodiversity maintenance and avoid to generate more production over requests, in order to support difficulties of fishery activities and integrate the needs of the market (Piccioli A., 2001).

Intensive aquaculture

Intensive aquaculture is a farming system based on the rearing of fish in artificial delimited spaces where human intervention is necessary for stocks management and feed administration. It is generally performed in inland tanks or in marine floating cages (Mipaaf, 2014, Pesca e acquacoltura).

Different system have been developed in intensive aquaculture:

1) Continuous system: is generally based on the use of several tanks, different in size and depth, in relation to the size and growth stages of reared fishes, named raceways, typical of trout and sturgeon farming (EU Commission- Aquaculture, 2015; Bronzi P. et al., 2001). The water supply is guaranteed by a channel that bring water from a river upstream and returns downstream after it has passed through all the tanks (Bronzi P. et al., 2001). Until the second half of XX century, this aquaculture technique was not fully developed, because it failed to guarantee an optimal alimentation and protection against infectious diseases (EU

Commission- Aquaculture, 2015). After that new technologies in pharmaceutical prevention of diseases and new formulations of feed improved productivity in intensive aquaculture. Feed adaptation to the particular needs of different species and different growth stages (larvae, juveniles and adults) favored large-scale production of rainbow trout from '60s and then of many other species in Europe. Other freshwater fishes, such as brown trout, brook trout, arctic charr, tilapia and Siberian sturgeon, represent now important productive realities (EU Commission- Aquaculture, 2015).

2) Recirculation system (closed system): is the most advanced system used now in intensive freshwater aquaculture. It is characterized by the use of recycled water that enters in tanks with a complex system of pipes (EU Commission- Aquaculture, 2015; Bronzi P. et al., 2001). The advantages of this methods are a complete isolation of thanks from external environment and that water parameters are kept under control (temperature, pH, salinity). Disinfection of water in this system is paramount, as well as wastes management before their release in the environment. Disadvantages of this system are high costs of maintenance and the dependence on an advanced technology (EU Commission- Aquaculture, 2015).

Recirculation systems gained success in countries with an extreme climate, where they can guarantee a constant temperature of water during seasons variation. Most reared freshwater species with this method are rainbow trout, catfish and eel, while the most diffuse saltwater fish is turbot (EU Commission- Aquaculture, 2015).

3) Floating cage system (open system): it is mostly diffuse in marine environments made in fixed or floating cages, that ensure a high fish density in relative limited spaces (Bronzi P. et al., 2001).

This system was born in Japan, with original floating support made of bamboo, and used for rearing of seabream and amberjack, and imported in Europe in '60s when was adapted to rainbow trout farming in Norwegian fjords (EU Commission- Aquaculture, 2015).

Around '70s and '80s this system was applied to the farming of Atlantic salmon, to gain weight after the smolt phase. Most of farms of Atlantic salmon are located in Nord Europe, mostly in Scotland and Norway.

In the southern Europe many countries adopted this system, firstly for seabream and seabass and recently for other species, such as meagre. During '90s developed many intensive aquaculture farms, in the Mediterranean area and in the Canary islands (EU Commission-Aquaculture, 2015). Greece registered an important development of this breeding systems for cultured seabass and seabream, particularly in central Macedonia (Piccioli A., 2001).

This farming system has been sometimes combined with shellfish production, in order to benefit from the purifying action of shellfish on waters coming from floating cages. The filtering capacity of the mollusks favors the elimination of fish nutrients wastes, but it also constitutes a limitation in the use of chemical substances in order to avoid their accumulation in filtrating apparatus of mussels and oysters (Mipaaf, 2014, Pesca e acquacoltura).

Intensive aquaculture of other marine flatfish species developed different systems, based on their habits to rest on a sandy bottom, with the use of tanks filled with sea water. During '90s, different coastal regions have taken advantage of this farming methods. Galicia (Spain) represented the most active reality of this aquaculture system for turbot and mussels. (Piccioli A., 2001).

Fixed cages have been used in developing countries for catfish and tilapia rearing and in some Mediterranean farm for seabream and seabass production. They are generally built with wooden or plastic materials and can be placed only in areas sheltered from winds and storm surges (Bronzi P. et al., 2001).

Floating cages are more flexible solution for both sheltered and more exposed areas, with a resistance to waves of 1-1.5 m. Fishes are kept inside a wide nest that is anchored to the seabed and kept afloat by a floating support (rectangular o circular), generally made in high

density polipolyethylene (HDPE).They can reach a volume of 4000 m^3 with a diameter of 10-24 m (Bronzi P. et al., 2001) (Fig. 1).

Cages with variable inclination can be use in surface or immersion. Their adaptability to different climate conditions make them suitable for areas subject to storm surges (Bronzi P. et al., 2001).

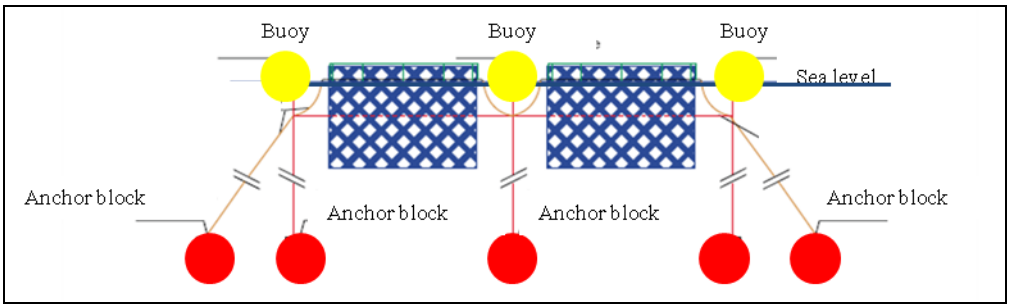

Fig. 1. Structure of a circular floating cage, lateral view. Anchor points (red dots) allow the anchor to the sea bottom. Floating buoys (yellow dots) keep cages at a depth of 2-3 m. (Bronzi P. et al., 2001, modified).

Extensive aquaculture

Extensive aquaculture is the closest farming methods to fishing (Cataudella S. et al., 2001). This aquaculture method developed to take the most of lagoons resources, first simply catching fishes and other aquatic products, then adopting more evolved techniques for capture and management of fish stocks in lagoon, diet supplementation and control of juveniles (Bostock J. et al., 2010).

Extensive and semi-intensive aquaculture systems represent now an important reality of fish production, particularly in the southern Europe. These farming techniques need large areas of water generally near the coastal areas. Typical environments are coastal lagoons, delta river, estuaries, bays and ponds of inland territories (Anras L. et al., 2010).

This kind of breeding is based on the use of extensive freshwater or brackish water basins that cover extended areas, mostly in coastal regions. Aquaculture in brackish water is defined as the breeding of aquatic species in waters with a level of salinity that varies from 0.5% to

seawater. These characteristics are typical of environments near the coastal area, such as estuaries, lagoons, bays, fjords. Generally only the last part of rearing takes place in brackish waters, since many cultured species may spend their earlier life in marine or freshwater (FAO, 1990-2015). Extensive aquaculture has an extremely important role in preserving these natural environments and their richness in vegetal and animal species. Many extensive aquaculture farming are regulated by minimal human intervention, typical of artisanal fisheries, as fixed capturing systems, nets or hand weapons. More evolved and specialized installations are regulated in different parts of productive cycle, like sowing, control of predators, diet integration and manuring interventions (Cataudella S. et al., 2001).

Productivity in extensive lagoons depend by the geographical characteristics of the farming, management ability and technical skills of operators above all (Cataudella S. et al., 2001). Traditional practices may present differences moving from one country to another, varying from protocols to water management (Anras L. et al., 2010).

General structures, that are normally present in fish farming located in lagoons, are hydraulic barriers as weirs and locks. In most cases fishing license is owned by one or few fishing cooperative that can manage and sell fish stocks coming from that lagoon. This model is a characteristic of traditional extensive aquaculture in lagoons (Cataudella S. et al., 2001).

Use of lagoons during last years has reached an optimal level and it has been estimated that there are over 10.000 ha of water surfaces dedicated to extensive use. Limitations to the expansion of extensive aquaculture are eutrophication processes and pollution risks. These problems caused a slow abandonment of marshlands and ponds in many countries such as Portugal, Spain and France (Anras L. et al., 2010).

1.1.2. Animal Production in extensive aquaculture

Global aquaculture production

Coastal lagoons are considered dynamic environments that link land to sea. They represent a complex ecosystem composed by a heterogeneity of species and characteristic ecological features (Pérez-Ruzafa A. et al., 2011). Lagoons present a high potential for human use, for their physical characteristics that protect this environment, such as a partial isolation from the sea.

These environments are exploited worldwide and over the past 30 years their production raised. In countries with a more temperate climate, such as Africa or Latin America, many fish species are reared in brackish waters (Bostock J. et al., 2010).

Lagoon environments are very important for fish production as they can host intensive and extensive aquaculture systems and also represent a privileged site for fisheries. Other destinations commonly reserved to lagoons are nautical sports or health care (Pérez-Ruzafa A. et al., 2011).

European aquaculture production

Extensive fish farming is the nearest form of aquaculture to fisheries, guaranteeing to the consumer a more natural and "wild" fish product. European aquaculture production is of good quality, that provides an excellent seafood products with high consumer protection standard, in accordance with animal health preservation principles and environmental sustainability (European Commission, COM (2013) 229 final). Differences exist in extensive aquaculture between various European countries, regarding territory characteristics, averages production and reared species (Anras L. et al., 2010).

Extensive aquaculture system in Portugal is mainly based on traditional farms constituted by micro enterprises (1380 farms) generally family based. Most of them are part of natural parks under law regulation and protection and for this reason limited to implement facilities.

Production are scarce and generally destined to local buyers. Moreover some areas are threatened by wastewaters from agricultural activities and urban areas (Anras L. et al., 2010). Clams production from intertidal areas have decreased due to the excess of organic matter, parasitic diseases (*Perkinsus marinus*) and the difficulties in managing predators. For these reasons clams production decreased 6 to 8 folds in 25 years (1980-2005). Annual shellfish production is estimated around 4320 tons. Other reared species are seabream, seabass, mullet, sole and eel (Anras L. et al., 2010).

In Spain there are many marshes and lagoons of high environmental value that were ancient salt ponds and were subsequently reconverted to aquaculture farms. They are part of natural parks and protected areas. An active effort by farmers is necessary here to protect embankments and ditches from destructive action of the sea. Shellfish production is the main sector of extensive aquaculture, with an average of 3000 tons/year, while fish, such as seabass, seabream and sole, are mainly reared with semi-intensive methods. Extensive aquaculture could further increase for the wide availability of spaces as in Andalusian costal lagoons (Anras L. et al., 2010).

In France semi-intensive and extensive aquaculture areas faced a decrease in production during past years, particularly in oysters and shellfish production, due to diseases outbreaks and unfavorable climatic condition (Anras L. et al., 2010). In some areas, such as Charente Maritime or Bretagne, shellfish farming represented an important occupational opportunity with an employing power of 4000 workers in the only Charente in 2000 (Piccioli A., 2001). Water quality, due to urban pressure and increasing population of the coasts, is one of the main problem in this aquaculture system and many research programs and engineering efforts have been made in order to improve water quality. Coastal marshes still posses available space for oysters storage and finishing but, although increased demand of the market, marshland didn't register increase in their use. Extensive fish farming is also part of French aquaculture. It developed on a surface of more than 4500 ha and it's mainly oriented to the

production of intertidal species, such as seabream, seabass, sole and mullets (Anras L. et al., 2010).

Greek extensive aquaculture is located in coastal estuaries. Seabream, seabass, sole, eel and mullets are the main represented species of fish farming, with an average production of 500 tons/year. There are many actions that can be undertaken to enhance productivity, such as the improvement of incoming watercourses, the creation of overwintering facilities for fish shoals and the management of macro-algal blooms with removal of vegetation (Anras L. et al., 2010).

The major limit to improvement of extensive aquaculture in Europe is the tendency to urbanization of these areas and urban, industrial and agricultural wastes that often represent a threat for the environmental safeguard (Anras L. et al., 2010). Data from different European countries demonstrate that aquaculture field can still expands and give new occupational opportunities in the EU and gradually satisfy consumer internal request of safe and sustainable seafood products (European Commission, COM(2013) 229 final).

1.1.3. Reality and Production of aquaculture in Italy

System of ponds and lagoons

Italian lagoons system covers an area of 100.000 ha, 40.000 of which are dedicated to extensive aquaculture, both in internal valleys and coastal lagoons. Many of them reach great extensions, from 5000 to 14000 ha (Sardinia, Veneto and Emilia Romagna) (Cataudella S. et al., 2001).

In the north of Italy (Veneto, Emilia-Romagna and Friuli-Venezia Giulia) extensive aquaculture is named "vallicultura" from the name "valli di pesca" that indicates coastal lagoons (Mipaaf- Pesca e acquacoltura, 2014). These environments suffer the ecological pressure exerted by aquaculture and agricultural activities, wastewaters from towns and industrial activities, that sometimes results in eutrophication of lagoons and ponds. Despite this threat for environmental safeguard, they still represent a reproductive site for many birds species that here can nest and also guarantee an abundant food source. Variable degree salinity and optimized use of these ecosystems provides a production of fishes and shellfish (Anras L. et al., 2010).

The main problem that "vallicoltura" is facing is the proliferation of piscivorous birds, particularly cormorants, that in the last 10 years have reduced the recapture of fishes of 30%. Moreover, fishes that escape from birds attacks can develop infections from wounds, further decreasing the aquaculture production (Anras L. et al., 2010; Cataudella S. et al., 2001). Other common problems caused by migratory birds are abnormal behavior in fish stocks during movement towards barriers or summer pasture, deformities due to scars from birds attack etc. (Anras L. et al., 2010).

Lesina and Varano lagoons represent important sites of extensive aquaculture in southern Adriatic coast. Located in the Gargano promontory, they cover surface of 5.136 ha and 6.100 ha, respectively. Fish capture here was historically performed by the use of "paranza", barriers

that anciently were built with reeds and in more recent times with nylon nests. These dams are used for conveying the fish towards the nets, mainly eels (Cataudella S. et al., 2001).

In the central Italy Tyrrhenian coast offers a variety of lagoons, in territories between Lazio and Campania, that are appreciated for their production and naturalistic value. Orbetello lagoon surrounds the peninsula from which takes its name and extends on an area of 2500 ha in the Argentario promontory. Aquaculture model is very similar to that applied in "vallicoltura" in the north of Italy, with the use of barriers and internal canals to collect fishes and basins for overwintering (Cataudella S. et al., 2001).

Fish production and species of interest

Italian production in extensive aquaculture is highly variable, depending on the heterogeneity of physiographic and on management characteristics of different installations. This system guarantees the 12,4% of national seafood production, with a medium production rates around 50 kg/ha, ranging from 40 kg/ha of Lesina lagoon to 319 kg/ha of Sardinian lagoons (Piccioli A., 2001; Mipaaf- Pesca e acquacoltura, 2014).

Although lagoon system possesses a valuable biodiversity and a richness in aquatic animals, only few species are of commercial interest. These generally include Mugilidae species, such as *Mugil cephalus* (grey mullet), *Chelon labrosus* (thicklip mullet), *Liza ramada* (thinlip mullet), *Liza aurata* (golden grey mullet), *Liza saliens* (leaping mullet). Other common species are *Sparus aurata* (seabream), *Dicentrarchus labrax* (seabass), *Anguilla anguilla* (European eel), *Atherina boyeri* (Boyer's sand smelt) (Cataudella S. et al., 2001).

Mullets represent the most important productive sector of extensive aquaculture as they account for the 58% of total, with an annual production of 3000 tons. Seabream and seabass are largely diffuse and constitute 16% and 13% of national production, respectively. Moreover eels complete lagoons production with a percentage of 13% (Mipaaf- Pesca e acquacoltura, 2014).

Analyzing production of different regions, "vallicoltura" proved to be an efficient and productive use of lagoon environments, with an annual production that varies from 30 kg/ha of Comacchio lagoon to 150.kg/ha of Veneto lagoon (Cataudella S. et al., 2001).

Lesina and Varano lagoons in the southern Adriatic recorded production lower than 40 kg/ha/year, mostly oriented towards eels farming, while Orbetello lagoons can reach 100 kg/ha/year of mullets, eels and seabasses and seabreams (Cataudella S. et al., 2001).

1.1.4. Extensive aquaculture in Sardinia

Extensive aquaculture in Sardinia can take advantage from one of the most extended area of lagoons and ponds of Europe. The exploitation of these territories started during nuragic age (XVIII-V cent. B.C.) and roman domain (X cent. B.C. – IV cent. A.C.), when were recorded the first clams producers, the "arsellari" from the Italian name of clam, "arsella" (Fenza A. et al., 2014).

Wetlands in Sardinia are represented by ponds and lagoons. These two terms are frequently interchanged although "pond" indicates a shallow basin with no apparent connections with the sea, while "lagoon" identifies a coastal basin separated from the sea only by thin land boundaries and with evident water efflux toward the sea (Fenza A. et al., 2014).

Production rates in Sardinian extensive aquaculture are estimated in a range, that varies from 50 to 150 kg/ha/year. Minimum data registered are 25 kg/ha/year, while the highest reached 325 kg/ha/year. This discrepancy is explained by differences between various farms. Each lagoon is managed by one or more cooperative of fisherman that owns the fishing license (Fenza A. et al., 2014).

Aquaculture here is mainly based on traditional methods. They exploit migratory behavior of fishes, particular juveniles that during spring enter lagoons, where they find abundance of food and a more protected environment than marine waters, and adult fishes that are attracted by this favorable source of food (Cataudella S. et al., 2001; Fenza A. et al., 2014). Based on this habits, between spring and summer removable barriers were placed to close passages between sea and lagoon, in order to entrap fishes. Now are present fixed fences, built in wood and reeds, that posses a typical V shape (Fig. 2). Recently new materials have been introduced, such as concrete and plastic, to facilitate management and improve resistance to weather insults (Cataudella S. et al., 2001; Fenza A. et al., 2014).

Figure 2. Typical wooden fences of extensive aquaculture in lagoons.(from Fenza A. et al., 2014)

In Sardinian wetlands is also diffuse a type of fishing that uses different portable tools, that are moved in different points of the lagoon, such as traps and nests, and are dropped tied together and retired after a short period. Fish species catches with this method are eels, gobies, crabs and cuttlefish (Fenza A. et al., 2014).

Sardinian products of extensive aquaculture are mainly based on few species, where mullets and eels are the most represented, but also crabs and sand smelts. Most prized species, such as seabreams and seabasses, are found in waters where salinity is higher, but also gobies, red mullets and flounders are captured in these environments. Shellfish also constitute an important products of extensive aquaculture, with different clams types as the most prized species (Fenza A. et al., 2014).

1.2. Parasites in Mugilidae

1.2.1. Protozoa

Protozoan can infect mullets both externally and in internal organs, causing diseases and even death. Protozoa represent a wide group, composed by several genera that share the single-cell form as common characteristic (Paperna I. and Overstreet R.M., 1981).

Among these parasites, *Amyloodinium ocellatum*, belonging to flagellates (Sarcomastigophora), has been reported in mullets, particularly in *Mugil cephalus* and itis generally considered responsible for outbreaks episodes (Paperna I. and Overstreet R.M., 1981; Galeotti M. et al., 2001). These parasites are not generally harmful for mullets that live in wide environments, while they can become a serious threat for fishes in confined waters, because their high reproductive cycle allows a rapid multiplication. After attachment to gill or tegument they undergo several division and can infest another host, resulting in heavy gill damage and consequent fish death (Paperna I. and Overstreet R.M., 1981).

Furthermore, the Ciliated protozoa (Ciliophora) are well represented in mullets, with *Trichodina* as the most cosmopolitan species (Paperna I. and Overstreet R.M., 1981; Öztürk, T., 2013). Ciliates live free in waters and soil and their transmission in fishes is generally direct by ingestion or cohabitation (Dykovà I. and Lom J., 2007). They are reported to infect many Mugilidae species, such as *M. cephalus* and *Liza aurata*. Target organs are gills and skin and parasites are generally found in low number, but under favorable conditions they become pathogenic. In crowded and delimited environments, as ponds during overwintering, this condition has been frequently reported (Paperna I. and Overstreet R.M., 1981; Dykovà I. and Lom J., 2007). *Trichodina*, due to its particular conformation characterized by one ring of interlocking denticles, is able to cut host epithelium. Young fishes are particularly susceptible to this parasitic infection that can cause their death (Paperna I. and Overstreet R.M., 1981).

A recent report on parasites of juvenile golden mullet has described *Trichodina lepsii* and *T. puytoraci* as the most common species in this Mugilidae. The first species was mainly found

in gills, while the latter was most present in skin and fins. The authors observed that the prevalence of infection was higher in winter season, probably due to eutrophication processes (Öztürk, T., 2013).

Mullets are intertidal species that can adapt to waters with different salinity degrees. Due to this particular characteristics they are exposed to pathogens both of marine and freshwater fishes. This is particularly evident with ciliates infections.

Other ciliated protozoan are *Ichthyophthirius multifiliis*, commonly known as "ich", that infect freshwater species, and *Cryptocaryon irritans* that is considered its marine counterpart. Both species have been reported in *M. cephalus* and other mullets. Pathogenetic mechanism of these two parasites are similar. They penetrate the host epidermis and establish in the vicinity of the basal membrane in the derma, where the growing process starts. After encystation parasites multiply and infect another host or move to other site of the same host. Common localizations are fins, gills and body surface (Paperna I. and Overstreet R.M., 1981; Burgess P.J. and Matthews R.A., 1995).

C. irritans can cause outbreaks due to the severe and diffuse damage to integument that results in osmotic imbalance. It is considered an acute disease that cause death after 5 days of exposure to theronts (infective forms) (Burgess P.J. and Matthews R.A., 1995).

1.2.2. Myxozoa

Myxozoa have been classified as protists until 1995, when they were assigned to Metazoa. They are considered primitively metazoan parasites that have secondarily degraded to parasitism. This phylum is constituted by two Classes: Malacosporea, that presents only a parasitic species for salmonids, and Myxosporea that infect fishes and annelids worldwide (Dykovà I. and Lom J., 2007b).

A characteristic of Myxozoa is the polar capsule provided with extrudable filament. Their reproduction is accomplished by an indirect cycle that have an annelid as definitive host and fish as intermediate host (Fig. 3). Spores, (myxospores) constituted by several cells, are characterized by the presence of a polar filament that use to attach to the intestine of the definite host, after its ingestion. In annelids spores proliferate and actinospores develop, with the presence of a plasmodial sporoplasm, that after attachment to the intermediate fish host, is released through the skin and migrates into the fish internal organs. Once inside fish body, sporoplasm transforms into trophozoites or vegetative stages, until reaches the formation of a sporogonic plasmodia, that is called pseudocyst and can expand until 2 cm in some species (Dykovà I. and Lom J., 2007b). Diamant in 1997 demonstrated that fish-to-fish transmission is possible by ingestion or by contaminated water. Myxosporidia feed by pinocytosis process or active transport in membrane surface (Diamant A., 1997).

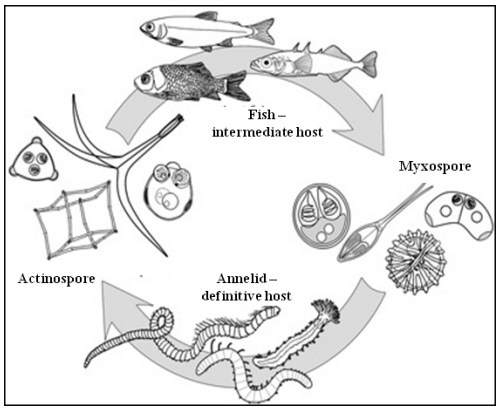

Figure 3 Life cycle of Myxosporea parasites. Spores and actinospores of different species display various morphologies.

Many myxosporean species have been reported in mullet, some of them causing massive outbreaks and are generally considered a threat for reared mullet (Paperna I. and Overstreet R.M., 1981; Paperna I., 1975).

In northern Black Sea were registered outbreaks attributable to *Myxobolus exiguus*, that infected gills and internal organs (Paperna I., 1975).

In mullets has been reported the presence of coelozoic (that infect natural body cavities) and histozoic forms (Paperna I. and Overstreet R.M., 1981). Colezoic forms, such as *Myxidium* spp, *Zschokkella* spp. and *Ceratomyxa* spp., were reported in gallbladder, urinary bladder and ureters of mullets although most of them don't cause severe harm to the host (Paperna I. and Overstreet R.M., 1981; Galeotti M. et al., 2001).

Most reported species of histozoic myxosporean are *Myxobolus* spp and *Kudoa* spp.. These parasites are frequently reported in brain, cartilages, internal organs and muscle (Paperna I. and Overstreet R.M., 1981; Paperna I., 1975; Roberts D., 2001). *Myxobolus* species probably represent the most often reported myxosporean parasite in mullets (Paperna I. and Overstreet R.M., 1981). They are found in gills, fins, mesentery, muscle of different Mugilidae species. Most reported species in mullets are *Myxobolus adeli*, *M. parvus*, *M. muelleri*, *M. ichkeulensis, M. spinacurvatura*, *Myxobolus rohdei*, *M. exiguus*, *Myxobolus nile*, *Myxobolus episquamalus* have been found in western Mediterranean (Spain) (Yurakhno V.M. and Ovcharenko M.O., 2014).

M. episquamalis has also been recently reported in *Mugil cephalus* from Senegalese coast and Korea (Diamanka A. et al., 2008; Kim W.S. et al., 2013). This parasite affects skin, but plasmodia has also been found in intestine, pancreas, kidney, stomach, heart, liver and spleen. Although they are normally not considered harmful for mullets, in case of unfavorable environmental conditions could elicit an epizootic (Diamanka A. et al., 2008)

Zschokkella admiranda has been recently described in *M. cephalus* for the first time in the Mediterranean fauna. *Sphaerospora dicentrarchi* and *S. sabrazesi* have been reported in *Mugil cephalus* and *Liza aurata* respectively (Yurakhno V.M. and Ovcharenko M.O., 2014). *Kudoa* species, such as *Kudoa unicapsula*, have been reported in muscle and alimentary tract of Mugil cephalus (Paperna I. and Overstreet R.M., 1981).

Polysporoplasma mugilis is a histozoic species that has been discovered in 1995 in kidneys of *Liza aurata* in the delta of river Ebro (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995). This parasite has also been found in other mullets, such as *Chelon labrosus* and *Liza ramada* (Yurakhno V.M. and Ovcharenko M.O., 2014) (Fig.4).

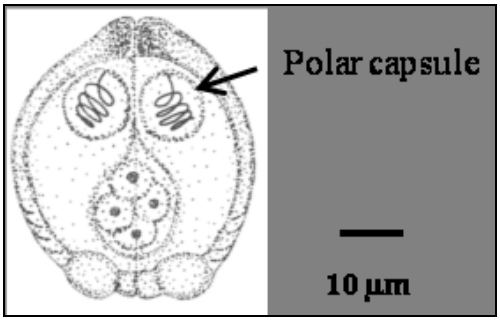

Figure 4. Spore of *Polysporoplasma mugilis*. Characteristic ellipsoidal shape, polar capsules (arrow) and size are keys for species identification (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995, modified).

1.2.3. Monogenea

Monogenea are ectoparasites that affect mullets on the external surface of the body, such as skin and gills, to which adhere through the use of hooks, anchors, suckers and clamps. Life cycle of these parasites is direct, as the larva, called oncomiracidium, hatches from an egg and infects another fish or continue its life on the same host. Numerous families of monogenean can infect mullets: Gyrodactylidae, Dactylogyridae and Microcotylidae (Paperna I. and Overstreet R.M., 1981).

Parasite of Monopisthocotyleans group comprises Gyrodactylids and Dactylogyrids. Both parasites infect several species of mullets and are reported to affect gills, fins and skin (Paperna I. and Overstreet R.M., 1981; Öztürk, T., 2013).

Dactylogyrids differ from Gyrodactylids for the presence of eggs rather than embryos (Paperna I. and Overstreet R.M., 1981).

Monogenean are often reported to affect mullets. Most frequently described species are *Ligophorus* spp, specially in *Liza* spp. This genus is closely specific to Mugilidae family. The presence of these parasites in *Liza aurata* have been recently reported. As other parasites, highest prevalence of *Ligophorus* spp has been registered in winter season and parasitic charge increased with size (Öztürk, T., 2013).

1.2.4. Digenea

Digenean parasites probably represent the most abundant group of parasites that affect mullets. These digenetic trematodes, commonly known as flukes, are endoparasitic flatworms that present an indirect life cycle, with more than two generation in different hosts (Paperna I. and Overstreet R.M., 1981).

The egg of a digenetic trematodes is generally released by a vertebrate host with the feces. It contains a larva, called miracidium, that infects the first host, generally a mollusk. Once inside mollusk, the miracidium undergoes replication of its germinal cells, that creates hundreds/thousands cercariae. Cercariae are larvae that encyst in the external part of the mollusk to be eaten by the second intermediate host (fish), where they transform into metacercariae. While the first mollusk host is generally specie-specific, the second intermediate host can be found in a wide range of vertebrates and invertebrates. After ingestion by the definitive host, that is comprised in a wide range of animals, metacercariae develop to maturity (Fig. 2). Mullets can be both intermediate or definitive hosts for many digenean parasites (Paperna I. and Overstreet R.M., 1981).

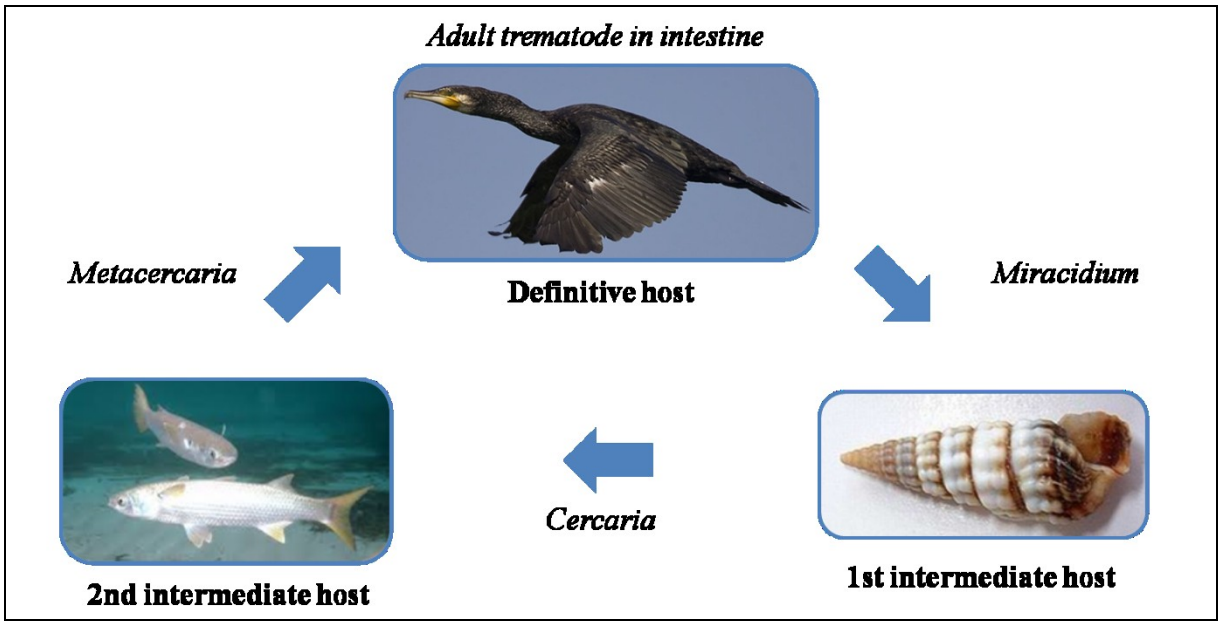

Figure 5. Digenean life cycle. Piscivorus birds here represent definitive host and mullets second intermediate host

Adults of digenetic trematodes are hermaphroditic worms that possess two muscular suckers, one in the anterior part of the body, named oral sucker, and one in the middle, the ventral sucker. They also have an intestine and inside their body few or thousand eggs can be visualized, depending on the species (Paperna I. and Overstreet R.M., 1981).

Most of the adult digenean that infect mullet as a definitive host belong to haploporids, haplosplanchnids and hemiurid (Öztürk, T., 2013). The first two groups generally have their metacercariae encysted in detritus or mud substrates, while hemiurids generally are transmitted by crustaceans and copepods as intermediate hosts (Paperna I. and Overstreet R.M., 1981). New species of the hemiurid genus *Saturnius* Manter, 1969 have been recently reported in *Mugil cephalus* from delta of Ebro river (Spain) and reclassification of other species has been done (Blasco-Costa I. et al., 2008; Blasco-Costa I. et al., 2006). Haploporidae species are largely diffuse in the Mediterranean and show a elevated family specificity to mullets (Culurgioni J. et al., 2014).

Harm and damage induced by adult trematodes is probably minimal for mullets in good environmental condition, when they are exposed to a balanced infective charged. Has been demonstrated that in ponds kept in poor conditions, when there is an elevated number of cercariae and their intermediate hosts, juvenile mullets can even die from a massive infection. For a long period mullets have been considered responsible of bringing haploporids from marine to freshwater environments, but this migration is probably due to the abundance of their intermediate hosts (mollusks) (Paperna I. and Overstreet R.M., 1981).

Digenean that use mullets as second intermediate hosts mostly belong to Heterophydae family, and infect definitive hosts such as aquatic birds and mammals and, less commonly, other fishes. While the binding between the snail (first intermediate host) and some heterophyd seems to be specie-specific, this is not true for mullets, which have been demonstrated to act as secondary intermediate host for many different digenean species. Young mullets seems to suffer from digenetic metacercariae infections, while in adults the

pathological changes associated to encysted larvae seems to be minimal (Paperna I. and Overstreet R.M., 1981).

Heterophyid trematodes represent a threat to public health, because they can infect man as definitive host after ingestion of raw or undercooked fish meat infected with metacercariae (Paperna I. and Overstreet R.M., 1981; Culurgioni J. et al., 2014). Most reported species are *Heterophyes heterophyes* and *Ascocotyle* (*Phagicola*) spp (Culurgioni J. et al., 2014), with a higher prevalence of *Ascocotyle* metacercariae during summer and autumn season and increasing prevalence with fish size (Öztürk T., 2013).

They are largely diffuse in the Mediterranean area (Paperna I. and Overstreet R.M., 1981; Culurgioni J. et al., 2014). As other digenetic trematodes they show a high specificity to Mugilidae (28). In a survey on mullets muscles, metacercariae of heterophyid were found in over 90% of examined fishes in Israel and almost 100% in the lake Manzala, in Egypt. Definitive hosts of heterophyid are represented by piscivorous birds and mammals. Dogs, cats and humans can be infected with metacercarie from fish (Paperna I. and Overstreet R.M., 1981).

Some heterophyid species can infect a wide range of definitive hosts, while others only few. *Heterophyes heterophyes* represents the most diffuse species in the Mediterranean sea and encysted in mullets and also as adult in many populations of fishermen or locals from Egypt near Nile delta. It has been reported a prevalence of 90% of infections in human in some areas that now are considered endemic. Dogs and cats of that area developed the infections of *H. heterophyes* as well, as they were fed with raw fish meat, but also showed the presence of other heterophyid that are not pathogenic for humans (Paperna I. and Overstreet R.M., 1981).

Symptoms of heterophyid infections in humans are most related to the alimentary system. They display abdominal pain, nausea, vomiting, diarrhoea and in the severe cases dysentery. Headache is frequently reported in association to this disease and complication of heterophyid

infection has been reported since eggs released from adults infecting intestine, where found in liver, heart, lungs and central nervous system (Paperna I. and Overstreet R.M., 1981).

1.2.5. Copepod

Copepods are crustaceans that can live free or parasitize many fish species, including mullets, where they can be found in gill arch, mouth and skin. Their pathogenetic relevance is due to their dimension. Generally males an some larval stages are free living, while female in some species are parasitic. Free living females are readily distinguished from parasitic females by the presence of modified appendages for the attachment to the host, such as claws (Ergasilids) or maxillipeds (Bomolochids) (Paperna I. and Overstreet R.M., 1981).

Many Ergasilids that infect mullets belong to the genus *Ergasilus.* They are characterized by small dimensions and the presence of antennae with which they can stick to gill filaments.

Caligids infecting mullets present a more heterogeneous provenience, as they belong to more than five families. Like many Ergasilids, they can infect a wide range of hosts, but generally prefer marine species.

Caligids infections start with the larval stages attached to a gill filament or elsewhere in the body in heavy infestations, by the use of modified antennae. Once fixed they undergo to 3-4 moults, until they reach the adult stage and take place in their definitive location, generally mouth or integument (Paperna I. and Overstreet R.M., 1981).

Many Copepods have been reported in mullets (Paperna I. and Overstreet R.M., 1981; Öztürk T., 2013; Plaul S.E. et al., 2013).

Their prevalence of Ergasilids species has been differently reported by authors. A recent report found the highest values in autumn (Öztürk T., 2013), while others in other seasons, probably due to different copepods species and different sites of sampling (Paperna I. and Overstreet R.M., 1981; Plaul S.E. et al., 2013).

1.3. Host-parasite interaction: Immune system and inflammatory response against parasites in Teleosts

1.3.1 Morpho-physiology of the immune system in Teleosts

Features of the immune system in Teleosts

Immunitary response is characterized by a non-specific response, a basic mechanism of defense that belongs to almost every living organisms and a specific one that is present only in vertebrates. The non-specific response is elicited by different pathogens and also by inorganic substances, while the specific response is directed against a molecular structure of the pathogen and provokes the activation of lymphoid system with the creation of an immunitary memory (Roberts D., 2001b).

Organs of immunitary system in Teleosts

Fishes lack the presence of bone marrow and lymph nodes. Kidney, spleen and thymus are the principal hematopoietic tissues in teleosts (Roberts D., 2001b; Dalmo R.A. et al., 1997; Noga E., 1997).

Kidney appears as a single elongated organ that generally lays ventrally to spine of fish (Roberts D., 2001b; Reimschuessel R. and Ferguson H., 2006). It can be divided in two functional parts, that are present in almost all fish: an anterior portion, that is the main hematopoietic tissue in teleostean fishes, and the posterior part that posses also excretory functions. Kidney is composed by endothelial cells, endocrine and excretory cells and various blood cell types in different maturation stages (Roberts D., 2001b, Dalmo R.A. et al., 1997; Reimschuessel R. and Ferguson H., 2006). Kidney receives an important blood supply, and along major vessels can be found cells with phagocytic activity. Here melanomacrophages centers, as in other teleostean organs, can increase in size and number after cachexia, chronic

inflammatory processes or scarce nutrition (Reimschuessel R. and Ferguson H., 2006; Agius, C. and Roberts R.J., 2003).

Spleen in teleosts seems to assume a role both in non-specific and specific immunity, ranging from cleaning macromolecules, hematopoiesis to antigen processing and antibody production. Its components are similar to those found in mammals: red pulp, white pulp, vascular structures and ellipsoids (Dalmo R.A. et al., 1997; Noga E., 1997). Red pulp is a complex structure of interlacing sinusoids and splenic cords that constitutes the main structure of splenic parenchyma (Noga E., 1997). Lymphoid cells of white pulp surround sinusoids, ellipsoids and melanomacrophages centers. Ellipsoids have an important role in spleen in trapping different substances, pathogens as well as old cells and protein aggregates. They are defined as sheaths of macrophages and reticular cells located around the termination of splenic arterioles (Roberts D., 2001b, Dalmo R.A. et al., 1997; Noga E., 1997). Splenic melanomacrophages are considered part of immunitary cells and play the same role of center of other organs. Melanomacrophages number seem to increase with age, exposure to stressor, pollution, diseases, also if these correlations haven't been proved in every case (Noga E., 1997; Agius, C. and Roberts R.J., 2003).

Thymus it's a paired organ located in the branchial cavity, characterized by lymphoid cells lined by a thin epithelium layer. Interesting characteristic in fish, is that its surface shows the presence of pores, and it can be readily exposure to antigen (Noga E., 1997). However it doesn't seem to have an active role in immunitary response against antigen, while it greatly affects T lymphocytes production, as demonstrated in thymectomized fishes, that showed an ablation of T-cell function (Roberts D., 2001b).

Liver has a minority role in teleosts immunitary system. It has a scavenging function and act as part of the reticulo-endothelial system, but Kupffer cells seems not to exist in teleostean liver (Dalmo R.A. et al., 1997; Evensen O., 2006). Melanomacrophages centers are commonly found in many fish species, characterized by the presence of pigment (melanin,

lipofuscin, haemosiderin). They are considered the piscine counterpart of mammalian germinal centers (Evensen O., 2006).

Heart role in fish immunitary system is still to be elucidated. Many studies showed that after an experimental injection of carbon particles endothelial cells showed a phagocytic capability in cod and plaice. Atrium endothelial cells seem to have a particular active role in phagocyting waste products (Dalmo R.A. et al., 1997; Poppe T. and Ferguson H., 2006).

Non-specific immunitary response

Non-specific immunitary response uses simple but effective methods to protect organisms from noxious agents. Surface barriers are physical obstacles, that act as first defense mechanism in teleosts (Roberts D., 2001b). They are represented skin, gills and gut, generally covered with a layer of mucus, that contains humoral factors with a toxic effect to pathogens, but that also acts as a physical impediment to many bacteria or parasites, entrapping them or inhibiting their adhesive power (Roberts D., 2001b; Dalmo R.A. et al., 1997).

Humoral factors contained in mucus act as growth inhibitors, lysins, precipitins and agglutinins. Growth factors are mostly represented by transferrin, that depriving microorganism of iron inhibits their growth, and interferon that exerts an anti-viral effect (Roberts D., 2001b). Lysins are enzymes able to induce cellular lysis of microorganisms. The complement is part of this category and acts as an enzyme cascade activating vasodilatation, attracts leucocytes and stimulates macrophages. Lysozyme in fish shows an higher capability to kill bacteria than in more developed vertebrates. It generally acts in conjunction with complement and it is directed against bacterial wall. C-reactive protein and lectins are proteins that in mammals are well known for their killing potential against bacteria. In teleosts they are known to be present but their role in the inflammatory process has not been still elucidated (Roberts D., 2001b; Dalmo R.A. et al., 1997).

Moreover epithelial and mucous cells can produce cytokines during monogenean parasites infection of skin (Mladineo I. and Block B.A., 2009; García-Castillo J. et al., 2002).

Non-specific defense mechanisms involves also cellular components of the immunitary system. Macrophages and neutrophils are cells involved in the phagocytic process. Phagocytic process is activated and regulated by two different molecules: opsonins and lymphokines. Opsonins can act both as non-specific and specific defense. The first option takes place with the complement, while the latter with antibodies.

The lymphokine that activate macrophages is the macrophage activating factor MAF, produced by fish T lymphocytes after exposition to the antigen (Roberts D., 2001b; Dalmo R.A. et al., 1997).

When the antigenic stimulus persists, macrophages go under further differentiation into epithelioid cells or giant cells. The effect of these cells is generally considered to be the same that occurs in mammals, with the ability to digest large particles or secrete toxic molecules, although in fish the real role of epithelioid cells and giant cells has not still been elucidated (Roberts D., 2001b; Ferguson H., 2006). Teleostean immunitary system and its response to noxious agents, seems to presents a biphasic answer. First arrive neutrophils that, after margination in blood vessel from head kidney, migrate in the tissue. Then macrophages, originated from circulating monocytes, arrive to the site of tissue damage. Monocytopoiesis process generally takes place in spleen and head kidney of teleosts (Reite O.B. and Evensen, Ø., 2006).

Specific immunitary response

Specific immune response in Teleosts is a response of specialized cells against antigens with a complex mechanism of antibodies, molecules and effectors cells (Roberts D., 2001b; Uribe C. et al., 2011).

Teleost posses different classes of antibodies, but probably the most diffuse is the tetrameric IgM that contains 8 antigenic sites. As in mammals, fish immune response is capable to acquire memory and is accomplished by a pool of memory B-cell. This is evident after a second exposure to the antigen. Cellular cytotoxicity is a mechanism present in several fish species, even if characterization of different cell type is still a difficult task for limits in molecular recognition (Uribe C. et al., 2011).

Acute inflammation

Fish do not seem to have an important acute cellular response if compared to mammals. The reason of this difference is still not clear, considering the fact that they possess the same cells of higher vertebrates (Ferguson H., 2006).

Cardinal signs typical of acute inflammation in mammals, *calor*, *rubor*, *dolor*, *tumor* and *functio laesa*, are not all present in teleosts, or at least difficult to differentiate. Fish are generally considered poikilotermic, which means that their body temperature is regulated by water temperature, although some large fishes, such as tuna, are known to possess a degree of thermoregulation. With this characteristic *calor* is not an assessable and useful sign to detect an acute inflammatory phase (Ferguson H., 2006).

Pathophysiology of inflammatory process in teleosts is similar to mammals. The acute phase is characterized by vasodilatation and leucocyte migration in the site of the trigger. Neutrophils arrive first at the site of inflammation, one hour after the inflammatory stimulus, then followed by macrophages and other inflammatory cells (Roberts D., 2001b)

Inflammation starts with vasodilator effects exerted by preformed amines (serotonine) on the circulatory system, followed by newly synthesized amines that attract leucocytes (30, 41). Once arrived at the site of inflammation, leucocytes release eicosanoids (arachidonic acid, prostaglandins, leukotrienes) that stimulates further vasodilatation, leucocytes attraction and

regulate other cells functions (Roberts D., 2001b; Uribe C. et al., 2011). In teleosts serotonin (5-hydroxytryptamine) replaces histamine in the inflammatory process.

Cytokines are low-molecular weight molecules that take part in fish immunitary response. They generally exert a local effect, but some of them, such as interleukin-1b (IL-1b) and tumor necrosis factor-α (TNF-α), can induce a systemic endocrine effect (Secombes, C.J. et al., 2001; Mladineo I. and Block B.A., 2010).

Once cytokines are released, cellular response takes few hours for the production of cytokines mRNA. It results in migration of neutrophils and macrophages to the inflammatory site.

It is known that in mammals they are produced by macrophages, lymphocytes, fibroblasts and epithelial cells (Mladineo I. and Block B.A., 2010).

TNF-α is produced by macrophages and is known to be a chemoattractant for neutrophils in teleosts (Roberts D., 2001b; Secombes, C.J. et al., 2001). Interleukin-1 (IL-1) is produced by several leucocytes but also by epithelial cells and stimulates lymphocytes proliferation and antibody production (Roberts D., 2001b).

Different forms of TNF-α have been found in fish, a costitutive one that is present both in healthy and parasitized individuals and another inducible group that is released during inflammatory processes (Mladineo I. and Block B.A., 2009).

Cytokines are involved in the inflammatory response against to different parasites groups, comprising protozoa, monogenea, digenea and copepoda. They are able to control parasite level and keep it at stable level because during parasite infection cytokines are upregulated (Mladineo I. and Block B.A., 2010).

Some examples of cytokines role in the inflammatory response against to different parasites are reported in case of *I. multifiliis* infection where it was observed that, IL-1b and TNF-α levels were higher from 4 to 26 days post infection and decreased after this period.

In another study regarding Digenea trematodes infection, cytokines expression was localized in lymphocytes and/or fibroblasts scattered around the cyst wall. It has been observed that

local Dydimozoid infection in gills doesn't stimulate a systemic cytokines response after local cytokines expression. Furthermore, cells secreting cytokines were identified also in the outer part of the cyst surrounding a Dydimozoid (Mladineo I. and Block B.A., 2010).

Different parasites, such as *Neoparamoeba* spp., elicit the cytokines response in cells that are directly involved in their attack, for example gill epithelial cells, while others cause a cytokines expression in cells that are outside the cyst in which the parasite is protected, as observed in Dydimozoid (Mladineo I. and Block B.A., 2010).

Macrophages are proven to produce cytokine "Transforming Growth Factor β2" (TGFβ2). This substance regulate macrophagic respiratory burst but there are also evidences that it can interact with endocrine system for the modulation of inflammatory response Acute phase is generally scarcely evident in fishes, and defense mechanisms of immune system are more effective in response to a chronic insult (Roberts D., 2001b).

Chronic inflammation

Chronic inflammation takes place when the acute process doesn't resolve the pathogenic insult and it's characterized by inflammation and proliferation of cellular components in surrounding tissues (Roberts D., 2001b; Ferguson H., 2006). Chronic inflammatory response in fish is very common and granuloma represents a general response against many noxious agents, such as foreign bodies, bacteria (*Mycobacterium* spp., *Renibacterium* spp., etc.), fungi and parasites (Roberts D., 2001b; Noga E.J. et al., 1989). After the acute phase the chronic inflammation takes place and generally a central area of necrosis is surrounded by inflammatory cells and macrophages that, maturing forward the center, create layers of flattened cells named epithelioid cells. Fibroblasts, present in mature lesions, produce collagen fibers and appear larger than fibroblast in normal connective tissue. Multinucleated giant cells, deriving from fusion of numerous macrophages, are scarcely reported in fish, but Timur et al. in 1977 described them in different fish species though their presence was probably temperature-

dependent. These cells appear when immunitary system encounters substances of difficult degradation (Roberts D., 2001b; Timur M. et al., 1997). A relevant feature in piscine chronic inflammation is the presence of melanin or other pigments that are generally detectable in cytoplasm of macrophages, named in these cases melanomacrophages (Ferguson H., 2006). These cells, that in normal condition can be observed in aggregates in many organs (spleen, liver, kidney), possess the capability to sequestrate foreign particles but also take part in encapsulating process. In some instances melanomacrophages presence can be massive and produces a typical black coloration. In skin of fish with metacercarial infection this phenomenon is so evident that takes the name of "black spot" disease (Ferguson H., 2006).

1.3.2. Cellular component of inflammatory response against parasites

Macrophages are part of the reticulo-endothelial system, disposed in different organs and particularly abundant in spleen, kidney and liver for their scavenging role. Macrophages play an important role in recognizing self from non-self antigens and also have an important function in antigen presentation both in non-specific and specific immunity (Roberts D., 2001b; Dalmo R.A. et al., 1997; Noga E., 1997; Secombes C., 1999).

Epithelioid macrophages, also known as epithelioid cells, took this name from their aspect resembling a cell of epithelial tissues in granulomatous formation. They are characterized by abundant cytoplasm with a glassy aspect and oval nuclei. Many studies with electron microscopy showed the presence of tight interdigitations of cytoplasmic membrane between adjacent epithelioid cells (Noga E., 1997; Noga E.J. et al., 1989).

In fishes epithelioid cells resemble characteristics of their mammalian counterpart. They show an active metabolism, as suggested by the presence of mitochondria and endoplasmic reticulum. Phagolysosome are present, testifying their closer nature with phagocytic macrophages (Noga E.J. et al., 1989).

There are evidence that epithelioid cells, previously considered macrophages cells, possess features of epithelial cells, such as well developed desmosomes with tonofilaments, as firstly demonstrated by Noga et al. in 1989 and then confirmed by other authors (Noga E.J. et al., 1989; Dezfuli B.S. et al., 2000). Mammalian and avian epithelioid cells do not posses desmosomes, which is considered the hallmark of typical epithelial tissues, and lack the expression of cytokeratin. In this study the expression of cytokeratin was confirmed by the presence in that cells of desmosomes. Moreover, although cytokeratin expression is not always evident in every fish species, the presence of this protein in immunitary cells involved in response to pathogens different in distant phylogenetically species, confirm the hypothesis that these mechanisms start from a common basis. In mammals the type of cell involved in the inflammatory response depend on the type of trigger that elicits immunitary response

(Ferguson H., 2006; Noga E.J. et al., 1989). For example, a study evidenced that in mammals, pathogens such as mycobacteria and fungi recruited mononuclear phagocytes that then differentiated in epithelioid cells, while injection of extraneous material, such as carrageenin, only involved mature macrophages. Timur et al. observed that after carrageenin injections flatfish developed an inflammatory response of epithelioid cells that showed interdigitation with adjacent cells but not desmosomes, as generally observed in this kind or reactions (Noga E.J. et al., 1989; Timur M. et al., 1997). In a study on brown trout naturally infected by the fungus *Saprolegnia* spp. Lopez-Doriga observed that in the late phase of the infection hyphae in the epidermis were surrounded by some cells, that they called "capsule cells" (CpCs), that apparently retarded or inhibited their growth and spreading (López-Dóriga M.V. and Martínez J.L., 1998). At ultrastructural examination they noticed that these cells possessed common features with filament-containing cells (FCCs), which are the constituents of epidermis in fish species. They concluded that these cells were not of leucocytic origin and were more probably belonging to the FCCs family (López-Dóriga M.V. and Martínez J.L., 1998). Other authors studying *Saprolegniasis* in thymus of fishes reported that phagocytic cells were mainly macrophages and thymic epithelial cells (49). They also speculated that some factors, maybe hyphae components (lipids and pl,3-glucans) could activate phagocytation. It is already known that in mammals and fish substances such P-glucans are able to modulate non-specific immunity, maybe with modulatory effect also on phagocytic activity of macrophages and epithelial cells (Álvarez F. et al., 1995).

Phagocytic power of epithelial cells has also been reported in mammals with *Histoplasma capsulatum* infection by human endothelial cells or tracheal cells of hamster (López-Dóriga M.V. and Martínez J.L., 1998). This fact has also been observed by Dezfuli et al. during a nematode infection in the European minnow *Phoxinus phoxinus* where epithelioid cells acted more as a barrier for nematode larvae than real phagocytic cells (Dezfuli B.S., 2000).

During inflammatory response against parasites, epithelioid cells are described like a corona of one or more layers of flattened macrophages that tightly surround parasite. At ultrastructural examination the inner layer of epithelioid cells shows finger-like projections (filopodia) that adhere to parasites and penetrate its cuticle. The cell contains many filaments, mitochondria and euchromatic nucleus. Cytoplasmic membrane had cellular projection apparently interdigitating adjacent cells with abundant desmosomes, composed by tonofilaments connected to 2 subplasmalemmar unit (Dezfuli B.S., 2000).

Immunitary system of invertebrates is only constituted by phagocytosis and encapsulating mechanisms that ensure the isolation of the pathogenic agent. It lacks leucocytes and immunoglobulins, which are present in vertebrates as fish. Macrophage is probably the first cell in the evolutionary process with phagocytic potential. Other leucocytes, that are well developed in mammals, probably first appeared in fishes during the evolutionary process (Reite O.B. and Evensen Ø., 2006).

Phagocytosis is an important defense mechanism that is highly conserved in each phylogenetic group of the animal kingdom. It consists in sequestration and destruction of foreign material or pathogens (Noga E.J. et al., 1989). In mammals phagocytes are macrophages and neutrophils and are distributed in various tissues, and after antigen activation they start to phagocytize extraneous material. Neutrophils are present in fishes, but their phagocytic power is less represented than in mammals and is mainly used against bacteria, as demonstrated in an experimental study. During piscine naturally occurring infections, macrophages have the most important role in phagocytizing pathogens, extraneous material, while neutrophils are more prone to kill pathologic agents by the secretion of enzymes and antimicrobial peptides in an extracellular location (Ferguson H., 2006).

In case of persistent antigen, macrophages can differentiate in epithelioid cells that surround the pathogenic material and isolate it from parenchyma. Encystation process is probably a primitive and nonspecific but effective mechanism for a quickly isolation of pathogens

developed in vertebrates, in which desmosomes of epithelioid cells assure a secure barrier and facilitate sequestration of agents (Noga E.J. et al., 1989).

Mesothelial cells, that posses cytokeratin, desmosomes and cytoplasmic interdigitations, normally presents in visceral cavities, can act as phagocytic cells in invertebrates animals, when they surround and encyst pathogens. In mammals normal mesothelial cells do not possess phagocytic activity, while when they undergone neoplastic transformation they can act as fibroblast and expressed tonofilaments and cytokeratin (Noga E.J. et al., 1989). A link between in the evolutionary process between mesothelial cells and phagocytic cells present in fish organs has been suggested, even if there are still no evidences to prove this connection (Dezfuli B.S., 2000).

Rodlet cells

Rodlet cells are enigmatic cells present only in fish. They have a peculiar aspect, characterized by the presence of cytoplasmic inclusions with a rod shape (from which they took their name) ad a central crystalline core (Fig.6). The existence of these cells was first reported by Thelohan in 1892, that described them as parasites present in fish tissues. In the following years and until recent times many authors confirmed this theory. These authors believed that were sporozoan and assigned them to the Apicomplexa group, under the name of *Rhabdospora thelohani* (Manera M. and Dezfuli B.S., 2004). The reason why these cells were considered parasites for a long time probably depend on the fact that they were not present in all fish species and frequently were not observed also in individuals of the same species. Moreover they possess capsule-like membrane and inclusions that resembled sporozoite stage of a parasites (Reite O.B. and Evensen Ø., 2006; Manera M. and Dezfuli B.S., 2004). After careful examination at electron microscope Paterson and Desser (1981) observed the presence of junctional complexes similarly to an epithelial cell. Moreover rodlet cells were always present in the epithelium in extracellular location and not intracellularly, as should be for an Apicomplexa parasite (Manera M. and Dezfuli B.S., 2004).

The different localizations of rodlet cells within the body observed between species could be due to an adaptation to the environment of each fish family. Has been hypothesized that could exist a "permanent" population of rodlet cell in organs constantly under pathogen attack and another "moving" population, that migrates to sites only occasionally invaded by pathogens (Reite O.B. and Evensen Ø., 2006).

Other arguments in support to the teleostean nature of rodlet cells are the lack of a tissue-specificity and the fact that they were reported also in young fishes and laboratory fishes kept under controlled condition (Manera M. and Dezfuli B.S., 2004).

Many authors, favorable to the hypothesis of teleostean origin of rodlet cells, thought them as leucocyte granular cells, while other researchers (Dezfuli B.S. et al., 2000; Reite O.B.,1997;

Dezfuli B.S. et al., 2003) hypothesized that they could be a glandular component of many epithelial tissues. They posses mithocondria very similar to those of epithelial cells of the same organ. Moreover, rodlet cells apparently start to develop from the basal layers of the epithelium moving forward to the surface, where they discharge their content, most likely a mucous cell (Manera M. and Dezfuli B.S., 2004), apparently contracting the fibrillar cytoplasmic border (Reite O.B. and Evensen Ø., 2006). Other functions ascribed to rodlet cells are an osmoregulatory function and ion transports (Dezfuli B.S. et al., 2000).

Most reports on rodlet cells describe their mature stage, but few authors investigated on the immature rodlet cells that appears with an oval shape, large pale nuclei and characteristic elongated mitochondria. During maturational process they develop the thick fibrillar capsule (Dezfuli B.S. et al., 2003). Once discharged their content, the capsule contracts and they go to apoptosis (Manera M. and Dezfuli B.S., 2004).

Two types of discharging mechanism have been observed for rodlet cells. The first occurs in rodlet cells of epithelia where they release their content by opening their apical portion into the lumen. The second mechanism happens in rodlet cells that are in the interstitial space of organs (liver, pancreas) and body cavity, by a lateral opening in the cytoplasmic membrane (Dezfuli B.S. et al., 2000).

The role of rodlet cells seem to take part in a later stage of the inflammatory response, when degenerative changes have already occurred, suggesting that probably they have a down regulatory role on the encircling activity of epithelioid cells. Rodlet cells also seem to be related to the presence of eosinophilic granular cells maybe related with the presence of chemical mediators. It is documented, by several authors, that during parasite infection of intestine or gills, rodlet cells appear at the site of infection after eosinophilic granular cells (Dezfuli B.S. et al., 2000).

Many recent papers reported the presence of rodlet cells during inflammatory response against different type of parasites, both metazoan and protozoan (Manera M. and Dezfuli

B.S., 2004; Dezfuli B.S. et al., 2007). Dezfuli et al. demonstrated an increased number of these cells in association to protozoan and metazoan parasites, specially near the inflammatory area or the site of attachment of the pathogen (Manera M. and Dezfuli B.S., 2004).

Many studies also highlighted the relation between a higher number of rodlet cells and stressing factors, such as water temperature changes, other medium condition, crowding in farming condition, toxicants and polluted water (Reite O.B. and Evensen Ø., 2006).

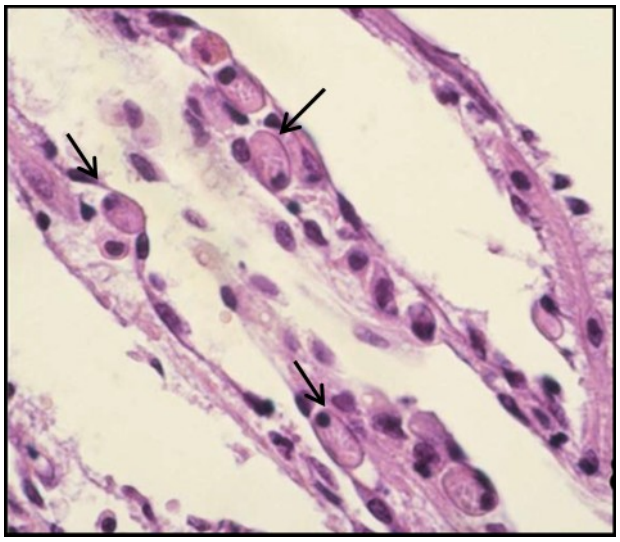

Figure 6. Rodlet cells (arrows) in the fish mesothelium. Rod shaped inclusions are parallel to cell long axis. (Reite O.B., 2005, modified).

Eosinophilic granular cells

Eosinophilic granular cells are cells belonging to the teleostean immunitary system and, as generally common accepted, they probably represent the progenitor of mammalian mast cells (Dezfuli B.S. et al., 2000). Eosinophilic granular cells posses common cytological features with mammalian mast cells such as multiple granules that show characteristic metachromasia when stained with toluidine blue or Alcian blue at low pH. With hematoxylin-eosin staining other granules appear eosinophilic and reveal their acidophilic nature (Reite O.B. and Evensen Ø., 2006; Reite O.B., 1997) (Fig.7).

Mammalian mast cells originate from bone marrow and then move into site of inflammation. In birds and mammals and human beings, mast cells are recruited in high number during inflammatory response against parasites at the site of attachment, as registered in many fish species. These findings, together with cytological characteristic and tissue distribution, reinforce the hypothesis that eosinophilic granular cells could be the progenitor cells of mammalian mast cells in the evolutionary process of vertebrates (Dezfuli B.S. et al., 2000).

These cells seem to be attracted in sites of inflammation caused by different agents, both of biological origin or not, as documented in salmonids and other fish species. Differences in tissue distribution have also been observed between various fish families. It seems that few eosinophilic granular cells are normally present in healthy tissues, while a high number of EGCs are recruited during parasites infection, suggesting that, as observed in rodlet cells, there is a "permanent" population of eosinophilic granular cells constituted by few cells and a "moving" one that is recruited only during inflammatory processes (Reite O.B., 1997).

Differing from mammalian mast cells, there is the lack of histamine in granules (Reite O.B., 1997), but they are known to contain other chemical mediators, such as alkaline and acid phosphatases, arylsulphatase and 5-nucleotidase, 5-HT, lysozyme and peptide antibiotics (piscidins) (Reite O.B. and Evensen Ø., 2006).

Some studies evidenced the role of EGCs in the inflammatory process. Dezfuli et al. in 2000 demonstrated that in European minnows infected with nematode parasites ECGs arrived at the end of the inflammatory process, after degenerative stages, suggesting a role in control and modulation of reparative process (Dezfuli B.S. et al., 2000). In the same way, as mammalian mast cells, the degranulation of these cells after reaction against pathogen produces vasodilatation and enhance the acute inflammatory reaction (Reite O.B. and Evensen Ø., 2006). Some studies on fish experimental infection with *Escherichia coli* and observation of the degranulation of eosinophilic granular cells, suggested that mediators contained in granules could have a chemotactic effect on neutrophils, as they arrive in the site of infection after EGCs degranulated. This mechanism result analogous to mammalian mast cells. An *in vivo* experiment showed that rainbow trout gill explants registered an higher number of EGCs than control group after inoculation of TNF-α and lipopolysaccharides (LPS) in the medium (Reite O.B. and Evensen Ø., 2006).

Mast cells in mammals were first discovered by Paul Ehrlich in 1877, that found these cells in the interstitial tissues around fibroblasts, with whom they were tightly close. From this observation he named the new cells "mast cells", from the verb "mästen" that in German means "to feed", because he thought that mast cells took their nutriment from fibroblasts. He was the first observer of the interaction between mast cells and fibroblast, that now is becoming a topic of interest because it probably very important for the pathogenetic mechanism of fibrosis (Hügle T., 2014).

During parasitic infection it has been observed that in some organs, that possess a communication route open towards the outside of the body, such as lungs or intestine, activated mast cells cause cough or diarrhoea, in order to eliminate parasites. When parasites infect other sites with no "escape" toward outside, such as liver or skin, they are encapsulated and blocked by a fibrotic reaction, a mechanism that seems to be elicited by mast cells alternative activation pathway. There are different theories on how this mechanism take place.

One of these, called "piecemal degranulation" consists in a slow release of mast cells cytoplasmic vesicles that enter lymphatic circulation from the interstitial space. Mast cells could activate immunitary response and even fibrosis with this paracrine/endocrine mechanism. It is indeed accepted that mast cells, not only possess receptors for TNF, but also represent a source of different cytokines and a reservoir of preformed TNF in human beings (Hügle T., 2014).

It has been hypothesized that mast cells can induce fibrosis by direct contact with fibroblast via gap junctions and induce collagen expression in fibroblast by secreting profibrotic molecules (cytokines and growth factors). They can also directly contribute to matrix formation, releasing proteoglycans (Hügle T., 2014).

In men infected with *Schistosoma* the formation of granulomas around eggs seems more to prevent host tissue injury caused by enzymatic activities of parasites than a measure to isolate and destroy the miracidium (Andrade Z.A., 2009).

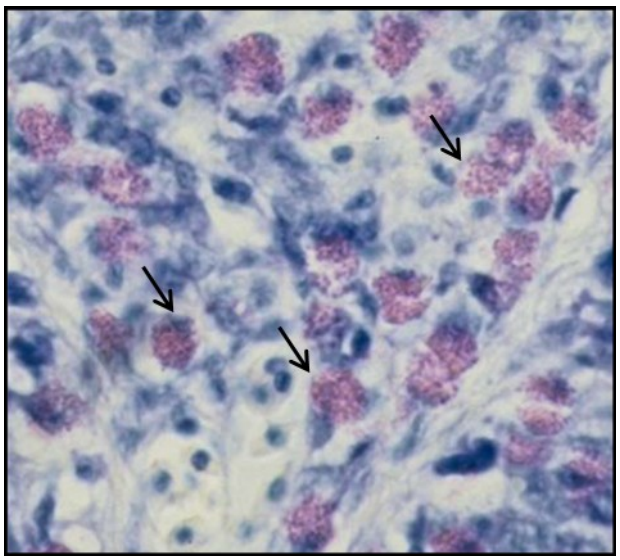

Figure 7. Eosinophilic granular cells (arrows) in the intestine of a fish with trematode infection. Metachromatic granules are stained with May-Grumwald Giemsa. (Reite O.B., 2005, modified).

Fibroblast

Fibroblasts are reported as integrative part of immunitary response to parasitic and bacterial disease. They act to contain and delimitate the parasite spread in conjunction with inflammatory cells or alone, as described in many reports of parasitic disease in different fish species (Roberts D., 2001b; Skorobrechova E.M. and Nikishin V.P., 2011). Some reports on acanthocephalan infection describe a capsule surrounding parasite entirely of fibroblastic origin, with the inner layers composed by mature fibrocytes and the outer part by plump fibroblasts producing collagen fibers. Parasitic response characterized only by fibroblast has been also reported in infection of digenetic metacercariae, although with some parasite species collagen production was not observed. In the yellowfinned sole infected with parasite *Corynosoma strumosum*, fibroblasts and collagen fibers were reported only in the outer layer in association to inflammatory cells, such as granulocytes and macrophages, that occupied the central part of the lesion. From these data fibroblasts seem to act in two different ways in teleosts in response to parasite infection. This difference seems to be related to the presence or not of an inflammatory response before the fibrotic reaction. In case of a host inflammatory response the role of fibroblast occurs only in a later stage, directed to delimitate parasite spread and to contain tissue damage (Skorobrechova E.M. and Nikishin V.P., 2011).

In some studies fibroblasts showed a phagocytic activity without the presence of macrophages or other inflammatory cells. It is the case of parasite *Didymocystis semiglobularis* infection in gills of tuna, where the reaction against this parasite consists in its encapsulation by fibrocytic cells only mixed with collagen fibers. This study also reported the presence of lysosomes inside the cytoplasm of fibroblasts and their phagocytic activity in some cases (Di Maio A. and Mladineo I., 2008).

2. Aim of the thesis

Parasitic diseases are widely diffuse in fishes. Parasites can exert a chronic harm on fish immunitary system and, even if they don't usually constitute a life threat for individuals, they could condition performances of fish population, specially influencing year class strength. This has been proved to be a risk for wild fishes and it can also be a limiting factors for aquaculture production.

Parasites, moreover, exert a role as biological indicators in aquatic environments and the study on their prevalence could be a useful tool to evaluate the health status of aquatic ecosystems.

To determine the influence of parasites on fish health is necessary to detect parasitic groups that affect a fish population and characterize the host-parasite interaction to understand the impact of these agent on fish immunitary system. For the aforementioned reasons the study of the present work has been mainly articulated in two phases:

- ✓ Investigate the prevalence of lesions caused by different classes of metazoan parasites in visceral organs of mullets (Osteichthyes: Mugilidae) from Sardinian lagoons;
- ✓ Evaluate type of lesions in parasitized organs and the response of fish immunitary system to pathogens by histopathologic and immunohistochemical characterization of lesions.

3. Investigation of the prevalence of parasitic disease in Mugilidae species in Sardinia

3.1. Introduction

3.1.1. Lagoons and Mugilidae breeding in Sardinia

Cabras Lagoon

Cabras lagoon is located in the Gulf of Oristano (Sardinia western coast) and is the most extended of the lagoon systems in Sardinia, as it covers 2380 h, belonging to Cabras, Nurachi and Riola Sardo towns (Obinu M. et al., 2008). It receives freshwater from surrounding wetlands and from Rio Mare Foghe (Lugliè A. et al., 2012). Bed of the lagoon is predominantly muddy and the depth varies from a minimum of 40 cm along the banks to 3 meters in the inner areas (Lugliè A. et al., 2012). Human intervention progressively changed the Cabras lagoon environment by the use of water drainage systems. (Fenza A. et al., 2014a; Obinu M. et al., 2008).

Intense agricultural activities and urban wastewaters conditioned status of eutrophication in the lagoon system (Lugliè A. et al., 2012). Cabras lagoon has always been considered a highly productive site for aquaculture and fishing. In 1998 productions reached 850 tons but decreased to 80 tons in 1999. This dramatic fall of productions was caused by an environmental crisis due to eutrophication process (Lugliè A. et al., 2012). After this episodes monitoring plans of water parameters such as salinity, temperature, pH, dissolved gas and phytoplankton were performed, as shown by Lugliè et al. in 2012. (Fig. 3.1). Salinity and water temperature underwent changes after 1999 and in subsequent years they didn't follow a regular trend. In 2008, following an intensely rainy autumn, water salinity decreased and temperature increased, favoring bloom of phytoplankton, particularly Cyanophyceae (Pulina S. et al., 2011). Southern part of the lagoon, nearest to the sea, presented higher levels of salinity (Comune di Cabras, P.U.C. 2011).

Although eutrophication processes constitutes a threat to environmental protection and aquaculture production, Cabras lagoon is now considered an oasis of wildlife protection and capture (Fenza A. et al., 2014a).

Capture techniques include both traditional and modern systems. In "Mare e' Pontis" area is localized a traditional weir made by wooden posts and reeds, but is also practiced fish capture by the use of nests (Fenza A. et al., 2014a; Obinu M. et al., 2008). The most represented fish species in the Cabras lagoon are mullets, eels, seabreams and seabasses.

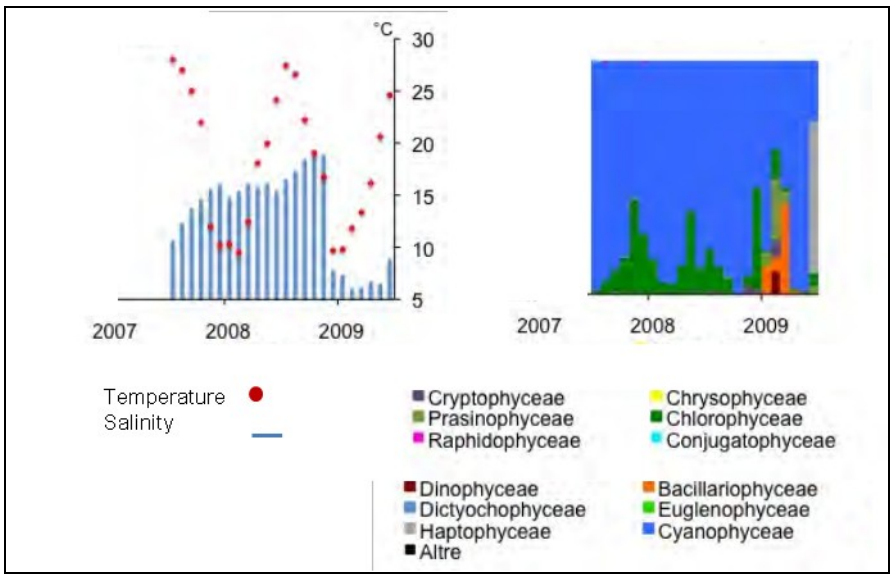

Figure 3.1. Analysis of salinity and temperature dynamics and phytoplankton composition. (Lugliè A. et. al, 2012, modified).

Marceddì-San Giovanni Lagoon

Marceddì-San Giovanni lagoon system is located in Gulf of Oristano and is constituted by Marceddì lagoon and San Giovanni pond, characterized by the presence of a deep cove. Marceddì lagoon, most external of two water basins, presents an extension of 900 h and is artificially separated by a barrier from the sea (Obinu M. et al., 2008).

Marceddì lagoon is divided between Terralba, Guspini and Arbus municipalities. Input of freshwater is guaranteed by affluent Rio Mogoro, Flumini Mannu e Rio Sitzerri. The

surrounding lands were used for mines, agricultural and breeding. Due to these activities, these waterways brought also mines processing residues, herbicides and fertilizers, leading to an environmental damage, limited only by sea water provision (Obinu M. et al., 2008).

Due to these disadvantages, during '90s, basins were built to flood drainage, another for freshwater collection and a riverbank to avoid direct input of freshwater from affluent rivers into the lagoon (Obinu M. et al., 2008). Fishes capturing here is made by direct catching, using artisanal and traditional techniques as nets, traps and longlines. Mainly fished species are sea bream and sea bass, mullets, eels and crabs. An important resource here is collection of mussels and clams (Obinu M. et al., 2008).

Marceddì lagoon is actually registered as a site of International convention for the protection of wetlands (RAMSAR) and it's an oasis of wildlife protection and capture (Fenza A. et al., 2014a).

San Teodoro Lagoon

San Teodoro lagoon, located in North-eastern Sardinia coast, belongs to San Teodoro municipality, from which is 1 km far (Fenza A. et al., 2014a; Comune di San Teodoro- Piano di gestione del SIC, 2014). Lagoon extends over an area of 3,5 km^2, delimited in the north by Punta Sabbatino and Lu Rattali in the southern side. This lagoon is constituted by the union of two basins, the proper lagoon (200 h) and "Pescaia" basins (30 h), that links San Teodoro lagoon to the sea. Lagoon beds are muddy in some areas or sandy, with rocks that reach water surface. Freshwaters provision is guaranteed by Rio San Teodoro, that represents the main tributary waterway (Comune di San Teodoro- Piano di gestione del SIC, 2014).

San Teodoro lagoon is located in two wealthy biodiversity regions (Gallura and Baronia), characterized by the presence of many vegetal and animal species of naturalistic interest. In this area many smaller lagoons are present, such as Porto Pozzo, Sa Curcurica, Avalè-Su Petrosu, that host colonies of local and migratory waterfowl (Fenza A. et al., 2014a). For

these reasons San Teodoro lagoon has become part of "Natura 2000", a system dedicated to protection of species at risk of extinction (Comune di San Teodoro- Piano di gestione del SIC, 2014).

Fish license has been accorded to a local cooperative, that manage fish capture trough the use of modern locks systems. Main fished species are mullets, eels, seabasses, flounders and breams. Shellfish production is articulated in clams culture, managed by another local cooperative, and oysters production, owned by a dedicated company that practices this culture by the use of floating structures (Fenza A. et al., 2014a).

Calich Lagoon

Calich lagoon is located in the northwestern region of Sardinia, the Nurra, in the municipality of Alghero. Nurra represents the Sardinian area in which lagoons are less extended and fish productive rates are lower compared to other wetlands of Sardinia(Fenza A. et al., 2014a). Calich lagoon, that presents a surface of 92 h, is now an area of naturalistic interest as it's registered as an oasis of wildlife protection and capture, belonging to the Natural Regional Park of Porto Conte (Fenza A. et al., 2014a,63).

Seafood production is quite varied, and most fished species are mullets, eels, sea breams, sea basses, salps, flounders and crabs. Fishing license is held by a local fishermen cooperative that sells its products (Fenza A. et al., 2014a).

From 2010 numerous episodes of anomalous discoloration of waters took place in Fertilia's coastal area, facing Calich lagoon. This fact suggested that the lagoon's tributaries, coming from inland territories, could have been involved in this phenomena (Arpas-Indagini sullo stato eutrofico dello stagno di Calich, 2011). Calich lagoon possess three waterways: the canale Urune that receives water from wastewater treatment plant of Santa Maria La Palma, the Rio Barca that brings waters from municipal sewage treatment plant located in "San Marco" area, and the Rio Calvia that carries water from inland cultivated territories

(vineyards, olive groves) (Fenza A. et al., 2014a, Arpas-Indagini sullo stato eutrofico dello stagno di Calich, 2011). In 2010 and 2011 water parameters such as salinity, temperature, pH, nitrites/nitrates, phytoplankton components and heavy metal concentrations, were measured from different selected point of the lagoon to evaluate correlation of these parameters with the aforementioned phenomena of water discoloration. Data evaluation revealed that this lagoon system suffered from nutrient provision deriving from nitrogen metabolism but mostly by phosphorus accumulation that was probably responsible for the microalgae development. Water discoloration in facing coastal areas appeared to be imputable to this microalgal bloom and the measurements of water parameters performed in Fertilia revealed similar phytoplankton components of Calich lagoon. Preventing overload of nutrient and phosphorus seems paramount for the maintenance of this delicate ecosystem, thus optimal functioning of depuration plants of wastewaters and careful reutilization of agricultural wastes are precautions that should be considered (Arpas-Indagini sullo stato eutrofico dello stagno di Calich, 2011).

3.1.2. Mugilidae species of interest

Mugilidae family, order Perciformes, are cosmopolitan fishes, distributed in all tropical and temperate seas (42oN to 42oS latitude), inhabiting inshore, lagoons and estuaries (Fenza A. et al., 2014a; Paperna I and Overstreet R.M., 1981; Boglione C. et al., 2006; Waltham N.J. et al., 2013). They represent an important food source in many world areas both for fisheries as well as aquaculture product (Boglione C. et al., 2006).

Mugilidae probably represent the most diffuse and captured fishes of lagoon ecosystem because for their euryhaline nature are capable to adapt in these environments characterized by variable salinity degree. This family is constituted by numerous species that inhabit seawaters, ponds, lagoons and also freshwaters basins (Fenza A. et al., 2014a; Paperna I and Overstreet R.M., 1981).

Most reported species in western Mediterranean belonging to this family are *Mugil cephalus* (flathead mullet), *Liza aurata* (golden grey mullet), *Liza ramada* (thinlip mullet) and *Chelon labrosus* (thicklip mullet) (Boglione C. et al., 2006).

Even if each species display peculiar characteristics, all mugilides present common features, such as fusiform body, slightly compressed at the sides, large scales and a grey coloration on upper back and silver grey along flanks. They have gregarious habits and their shoals can reach considerable size (Fenza A. et al., 2014a).

Mullets have the capability to get feeding from water surface to mud bottom, even if each species has developed a particular adaptation to environmental characteristic and utilization of different substrates (Fenza A. et al., 2014a, Boglione C. et al., 2006). This adaptation is reflected in the characteristics of oral and pharyngeal anatomical structures . *Chelon labrosus* usually feeds on organic debris and algae by using tubercles of its upper lip, while *Liza* genus prefers soft mud with smaller particles. *Mugil cephalus* is generally attracted by more larger particles and sandy substrates (Boglione C. et al., 2006).

Differences between various Mugilidae species are also present in the spawning season, but generally reproduction takes place in the sea, as eggs and larvae are pelagic (Fenza A. et al., 2014a). Larvae feed on zooplankton. After this phase fry of the same species take part to a school in areas near to the coast and estuaries when they are around 2 months old (Boglione C. et al., 2006).

Liza aurata

Liza aurata (Risso, 1810), (Order Perciformes, family Mugilidae, genus Liza) commonly known as golden grey mullet, is a component of Mugilidae family and one of the most appreciated mullets. The name "aurata" is referred to the characteristic golden spot present on the operculum (Fenza A. et al., 2014a; Fishbase- Liza aurata, 2015). Body is elongated and thin, with a slightly flattened head. Mouth is small and lips are well developed. Other morphologic recognizable features of *L.aurata* are long pectoral fins (that lack black spot at the fins base), dorsal spines in number of five, 7-9 dorsal soft rays and 3 anal spines. The body is generally covered by a thick mucous layer (Fenza A. et al., 2014a). Females can reach a maximum length of 34 cm and males 59 cm, although common length generally doesn't exceed 30 cm (Fishbase- Liza aurata, 2015) (Fig. 3.2.).

As other mullets *Liza aurata* is considered a catadromous species that spawns in the sea, generally from July to November. Females progressively reduce ovary size, while in males testicular volumes increases with age. Fecundity of this species is similarly to other Mugilidae species in a range from 80.000 to 1.400.000 eggs (Hotos G.N. et al., 2000).

Fry mainly feed on planktonic forms such as *Brachionus urceolaris* group, *B. quadridentatus* and *Colurella* sp.. Salt-water organisms such as polychaete larvae and the cladoceran *Podon polyphemoides* are frequently found in fishes caught in the morning. They feed during the day but predominantly in the morning (Tosi L. and Torricelli P., 1988).

Adults generally moves into crowded schools and enter estuaries and lagoons (Fishbase- Liza aurata, 2015). *L.aurata* prefers waters with a higher degree of salinity and present a more pelagic attitude compared to other Mugilidae species (Fenza A. et al., 2014a; Arechavala-Lopez P. et al., 2010). This species if frequently observed near intensive aquaculture floating cages, were waste nutrient of reared fishes is readily available (Arechavala-Lopez P. et al., 2010).

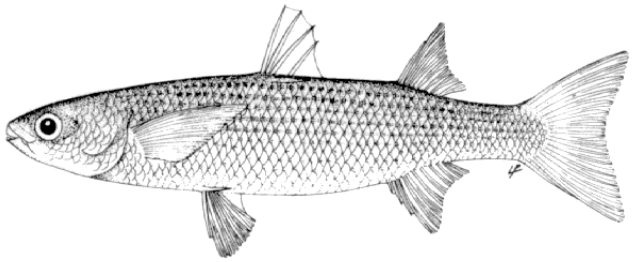

Figure 3.2. *Liza aurata*. (fishbase.org)

Liza ramada

Liza ramada (Risso, 1827) (Order Perciformes, family Mugilidae, genus Liza), commonly named "thinlip grey mullet", is a family member of Mugilidae and largely diffuse in the Mediterranean area. It is commonly found along coasts and in estuaries and lagoons, where prefers environments with lower salinity and temperatures between 8-24 C°. It also can be found in rivers and freshwater basins (Fenza A. et al., 2014a).

Body is elongated and thin, with a relatively massive but sharp head profile (Fenza A. et al., 2014a; Fishbase- Liza ramada, 2015). Important anatomical features are found on the head and fins. Upper lip is smooth and adipose eyelid is absent. Pectoral fins are short and don't reach the eye if folded towards the head (Fenza A. et al., 2014a). Dorsal fins display 4-5 spines, 7-10 dorsal soft rays. Anal spines are generally 3 and anal soft rays can vary from 8 to 9 (Fishbase- Liza ramada, 2015) (Fig. 3.3.).

Common body length generally can reach 35 cm, even if have reported specimens of 70 cm.

Adults of *L.ramada* are pelagic and enters lagoon or rivers in large schools. They feed on algae, benthonic organisms or plankton. Spawning season occurs from September to February when mullets move to marine waters near the coasts. *Liza ramada* fry and juveniles principally feed in plankton, as it constitutes 50% of their diet. Other constituents of their diet are benthonic organisms. Prey are mainly represented by small freshwater species (Tosi L. and Torricelli P., 1988; Fishbase- Liza ramada, 2015). Adults mainly feed on extensive areas of mud, benthonic micro algae, organic particles (Almeida P.R., 1996).

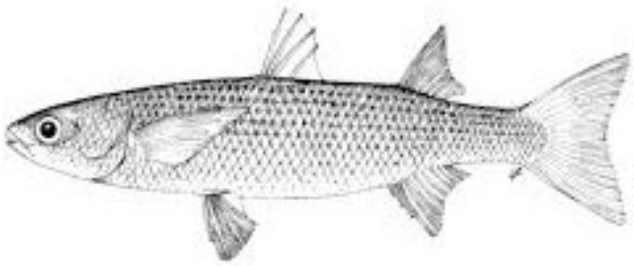

Figure 3.3. *Liza ramada*. (fishbase.org)

Chelon labrosus

Chelon labrosus (Risso, 1827), (Order Perciformes, family Mugilidae, genus Chelon), owes its name "labrosus" to its developed upper lip and that therefore it is commonly called thicklip grey mullet (Fenza A. et al., 2014a; Fishbase- Chelon labrosus, 2015). *C.labrosus* possesses an elongated body that in transversal section appears almost circular in shape. Even if upper lip is a distinctive feature alone, other morphologic characteristics are the presence of 5 dorsal spines, 7-9 dorsal soft rays, 3 anal spines and 8-9 anal soft rays. In this species female are larger than males; they can reach 75 cm in length, but common size generally doesn't exceed 32 cm (Fishbase- Chelon labrosus, 2015) (Fig. 3.4.).

Adults enter in lagoons or freshwaters with muddy or sandy beds, that prefer. Their shoals present continuous migratory habit form seawaters towards the lagoon for the research of food (Fenza A. et al., 2014a; Arechavala-Lopez P. et al., 2010). They feed on diatoms, algae, small

invertebrates such as molluscs and crustaceans. Spawning season goes from January to March (Fenza A. et al., 2014a; Fishbase- Chelon labrosus, 2015).

Chelon labrosus fry feed mainly on planktonic, specially rotifers, and benthonic organisms, such as oligochaetes, larvae of chironids and brackish water copepods. Feeding activities is mainly focused in the morning hours (Tosi L. and Torricelli P., 1988).

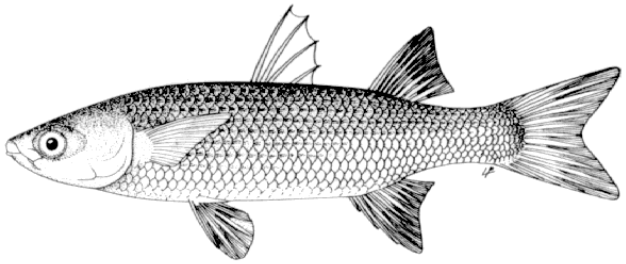

Figure 3.4. *Chelon labrosus*. (fishbase.org)

Mugil cephalus: a species of high commercial value

Mugil cephalus (Linneo, 1758), (Order Perciformes, family Mugilidae, genus Mugil), also known as grey mullet, striped mullet or sea mullet, is probably the most common and cosmopolitan member of the Mugilidae family (Waltham N.J. et al., 2013; Whitfield A.K. et al., 2012). Body is elongated but solid, almost cylindrical in its central portion (Fenza A. et al., 2014a). The head is large and flattened, lips are smooth and thin and the eyes present a characteristic adipose capsule that cover most of the pupil (Fenza A. et al., 2014a; Fishbase- Mugil cephalus, 2015). Pectoral fins present a dark spot on their base. Dorsal fins are in number of 5, soft dorsal rays can vary from 7 to 9, anal spines are 3 and anal soft rays 8-9. Maximum reached length is of 100 cm, but common average size is general lower than 50 cm (Fig. 3.5.).

As *M.cephalus* is a gregarious fish, adults are often found in large shoals in estuarine and marine coastal areas. Muddy or sandy bottoms are preferred by this species, as *M.cephalus*

mainly feeds on detritus, algae and benthic organisms during the day (Fenza A. et al., 2014a; Fishbase-Mugil cephalus, 2015).

Juveniles under 25 mm in length are generally planktonic and carnivorous, and as they increase in size their alimentary habits change into detritivorous. During their life as fry they predominantly feed during morning or sunset on benthonic forms such as nematodes, oligochaetes and larvae of chironomids (Tosi L. and Torricelli P., 1988).

Spawning season goes from August to October in the sea. Females can spawn up to 2.6 million of eggs.

M.cephalus represents the most appreciated species among Mugilidae family, both for direct consumption and for its derived products Fenza A. et al., 2014a). Their fillet is appreciated for its taste and because contains a large amount of high protein and vitamins (Whitfield A.K. et al., 2012).

This species, for size and characteristic of its ovaries, is preferred among other mullets for the production of fish rows (bottarga) (Fenza A. et al., 2014a; Whitfield A.K. et al., 2012). It's made by salting and drying of ovaries that have been collected at the end of summer. This product is of high commercial value and it's an ancient techniques still preserved in Sardinia and other parts of Mediterranean area (Fenza A. et al., 2014a).

Figure 3.5. *Mugil cephalus*. (fishbase.org)

3.1.3. Parasites and main affected organs in mullets

Digenean metacercariae

Digenetic trematodes have been widely reported in mullets andmany digenean metacercariae in fishes have the ability to infect a wide range of mullet as intermediate hosts. . The complete life cycle of these parasites has not been elucidated in all species (Simões S.B.E. et al., 2010). The most diffuse digenean that infect Mugilidae species in the Mediterranean area are Heterophyids, that represent a zoonoses (Ghobashy M.A. et al, 2010; Elsheikha H.M.and Elshazly A.M., 2008). Frequently reported species in Mediterranean brackish waters are *Heterophyes* sp., *Stictodora* sp., *Phagicola* (Ascocotyle) (Masala S. et al., 2014).

Digenean metacercariae are generally observed encysted in muscle, but they can be found in almost every organ of mullets. They have been in fact reported in liver, spleen, kidney, eye, brain, heart, etc (Ghobashy M.A. et al, 2010; Elsheikha H.M .and Elshazly A.M., 2008; Martorelli S.R. et al., 2012; Kotb H.L. et al.,2014 ;Öztürk T., 2013).

In stained section encysted metacercariae appear as circular structures with a twisted metacercaria inside. The outer layer is constituted by a cyst wall that protects parasite from host inflammatory reaction. In many sections is possible to appreciate oral or ventral suckers, characterized by a musculature disposed in a radial way. From oral sucker departs a muscular pharynx that continues in and paired caeca, appreciable in some histological section (Bruno D.W. et al., 2006).

Myxobolus spp.

Myxosporean parasites have been extensively reported in Mugilidae species worldwide. The most described species is *Mugil cephalus,* to which were attributes 36 myxosporean species. Golden grey mullet (*Liza aurata*) has been reported as host of 18 different species, while *Liza ramada* and *Chelon labrosus* of 9 and 6 respectively. Myxobolidae family represent the most

reported group of myxosporean parasites in mullets. The genus *Myxobolus* alone includes 32 species (Yurakhno V.M. and Ovcharenko M.O., 2014).

Myxobolus exiguus (Thélohan, 1895), has been reported in many Mugilidae species (*C.labrosus*, *M.cephalus*, *L.aurata*, *L.ramada* and *L.saliens)* from Mediterranean area and Caspian sea and Atlantic ocean, to infect gills, pyloric caeca, heart, intestine, kidney, spleen and gall bladder (Yurakhno V.M. and Ovcharenko M.O., 2014).

Myxobolus muelleri (Butschli, 1882) has been observed in Atlantic ocean, Black Sea and Mediterranean. *L.ramada*, *L.aurata* and *M.cephalus* showed its presence in gills, intestine, urinary bladder, liver, kidney, spleen, heart, muscle and gonads.

M.cephalus from delta of Ebro river has been reported as host of *Myxobolus rhodei*, that has been observed in many internal organs, such as kidney, gall bladder and intestine, and in muscle (Yurakhno V.M. and Ovcharenko M.O., 2014; Kent M.L. et al., 2001).

Myxobolus spinacurvatura has been reported in many sites of Mediterranean area in *M.cephalus*. Main affected organs were intestine, liver, gall bladder and spleen (Yurakhno V.M. and Ovcharenko M.O., 2014; Kent M.L. et al., 2001).

Many other species belonging to the genus *Myxobolus* have been described in mullets in the Mediterranean basin and worldwide (Yurakhno V.M. and Ovcharenko M.O., 2014; Yemmen C., 2011; Al-Bassel D.A. et al., 2010).

Many myxosporidian spores can be visualized in histopathologic sections, even if Hematoxylin-Eosin stain do not show details. . Spores are acid fast (Ziehl-Neelsen) and their staining intensity increases as they go toward maturation, while polar capsules stain in blue-black (Bruno D.W. et al., 2006; Nowak B. et al, 2002).

Polysporoplasma mugilis

*Polysporoplasma mugilis (*Phylum Myxozoa, Class Myxosporea, Order Bivalvulida, suborder Variisporina*)* is a myxosporean parasite of mullets that belongs to Polysporoplasmidae family (Yurakhno V.M. and Ovcharenko M.O., 2014). It has been firstly reported in 1995, infecting *Liza aurata* adults and juveniles from delta of river Ebro. Reported prevalence was 14,8% in the examined population (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995). It is a histozoic parasite with a specific target organ, the kidney, where it can be found in sporogonic cysts inside glomeruli, tubules or in the interstitial connective tissues. Single spores of *P. mugilis* measure 25 Lenght x 21 Width mμ, thus sizes and the characteristic ellipsoid shape allow an easy distinction from other myxosporean forms. Its name, Polysporoplasma, derives by the presence of more than two sporoplasm in the spores, an unique features among myxosporean genera. It's still not clear if each sporoplasm can start a new life cycle as well as the presence or identity of intermediate hosts (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995). *P.mugilis* has been reported in other Mugilidae species, such as *Chelon labrosus* and *Liza ramada* from Mediterranean sea and *Liza aurata* from Black Sea (Yurakhno V.M. and Ovcharenko M.O., 2014).

3.2. Material and methods

3.2.1. Sampling and species identification

Mullets (Osteichytes: Mugilidae) were collected from four different sampling sites in Sardinia: Calich lagoon, San Teodoro lagoon, Cabras lagoon and Marceddì lagoon. From each site 30 fishes (expet a sampling from Marceddi lagoon where fishes were 29) were collected in summer sampling (July 2014) and in autumn (October 2014) for a total number of 239 fishes collected . Species recognition was accomplished by the use of taxonomic keys widely described in literature.

3.2.2. Necropsy

Fishes were euthanatized by overexposure to Tricaine Methanesulfonate (MS-222). After carefully evaluation of the presence of macroscopic lesion on body surface, each fish was disposed in right lateral recumbency. An incision was made on the mid-line cranially to the pelvic girdle to avoid fecal contamination from the intestine, and moving forward to the ramus of mandible. Another incision has been made from the pelvic girdle moving dorsally and then cranially to reach the operculum. The abdominal wall and the operculum have been removed. Viscera were removed and samples of heart, livers, spleen and kidney were collected. Specimens were then identified with a code and placed in cassettes for tissue processing. Gross pathological patterns were registered and digital pictures were collected.

3.2.3. Parasitological examination

Further fishes were sampled from each lagoon and submitted to parasitological examination. Target organs (heart, liver, spleen and kidney) were put on a Petri dish to search endoparasites.

Metacercariae have been examined with the technique of picric acid. After GAP solution preparation (glicerin - picric acid 1:1), metacercariae have been placed on a slide with a drop

of 0,9% saline solution, covered with a cover slip and put a drop of GAP at the side of the cover slip (diffusion by capillarity). Obtained specimens have been evaluated for the presence of morphometric features (wall thickness, length and width of metacercarie, excised parasite, oral, ventral and genital sucker, pharynx, prefarynx and esophagus length), to classify metacercariae at genus level.

Myxosporids were searched by fresh smear preparation and then evaluated at light microscope.

All parasites have then been photographed by the use of a digital system of micro photo, measured, registered by taxon and organ localization.

Parasites identification has been made taxonomic level closer to the species by the use of taxonomic keys.

3.2.4. Histopathology

Samples of target organs (liver, spleen, heart and kidney) were 10% formalin fixed for 48h dehydrate with increasing concentrations of alcohol and xylene in an automatic tissue processor and, finally, paraffin embedded. Sections of 3µm were obtained with a microtome (RM2245, Leica Biosystems). Sections were then stained in an automatic multistainer (ST5020, Leica Biosystems) with hematoxylin-eosin (HE) and with Ziehl-Neelsen staining. Specimens were then evaluated at light microscopy (Nikon Eclipse 80i). Microphotographs were taken and lesions were classified on the base of etiological agent.

3.2.5. Data collection and statistical analysis

Fish species, site of collection, organ and type of parasite were registered and elaborated to evaluate abundance and prevalence of examined specimens.

3.3. Results

3.3.1. Necropsy

Necropsy revealed the presence of macroscopic lesions in liver, spleen and heart in 66/239 (27,6%) of collected mullets. Kidney did not show any significant macroscopic changes. Morphological alterations were mainly nonspecific, comprising splenomegaly, hepatomegaly and steatosis. Few hemorrhagic lesions were found in heart and spleen (Table 3.1.).

Furthermore , small whitish nodular lesions, ranging from 0,5 mm to 2 mm in diameter, protruding from the rest of parenchyma and with defined borders, were found in spleen, liver and heart.

Main lesions observed at necropsy are illustrated in Fig. 3.6

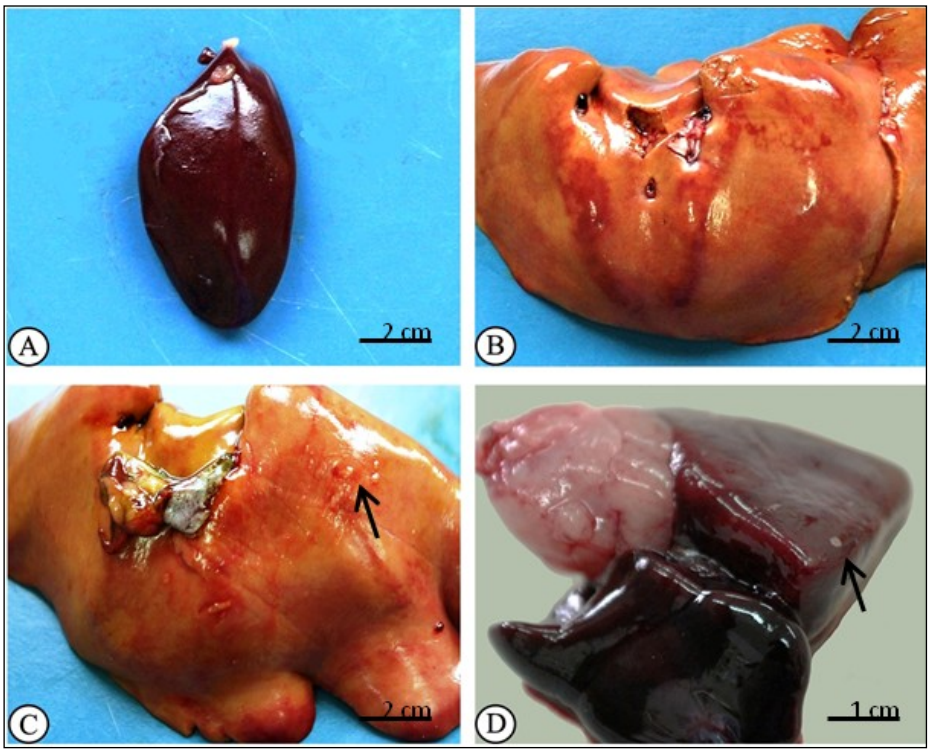

Figure 3.6. A-D. Main macroscopic lesions observed in visceral organs of Mugilidae species.
A. Spleen**.** Splenomegaly with rounded edges and increased size. **B.** Liver. Increased size (hepatomegaly) and friable and greasy appearance. **C.** Liver. Multiple nodular lesions (arrow) are visible on serosal surface. **D.** Heart. Single whitish and well defined nodule protrude on ventricular wall.

	Liver	Spleen	Heart
Splenomegaly / Epatomegaly	5/66	15/66	—
Steatosis	42/66	—	—
Hemorrhagic lesion	N	2/66	1/66
Nodular lesion	12/66	13/66	5/66

Table 3.1. Main lesions observed macroscopic examination. Liver steatosis has the highest prevalence among non-specific pattern. Spleen and liver were the most affected organ by nodular lesions. N= undetected.

3.3.2. Parasitological examination

The parasitological examination showed the presence of 3 genera of metacercariae (larval stage of metazoan parasites belonging to the class of Digenean, Phylum Plathelmintes): *Heterophyes* sp., *Stictodora* sp., *Phagicola* (*Ascocotyle*) sp. (Fig.3.7. A-C)

Metazoan parasites belonging to the phylum Myxozoa were identified in mullets from each lagoon. Spores and sporogonic cysts were found in liver, spleen, heart and kidney (Fig.3.7. D-F). They were attributed to the genus *Myxobolus* spp.

Another myxosporean parasites has been observed only in kidney in glomeruli, tubules and interstitial tissue of trunk kidney (histozoic). Mature spores were spherical to ellipsoidal, measuring 25 μm length x 21 μm width. Due to host species, morphological identification features and organ localization, this myxosporean has been assigned to the species *Polysporoplasma mugilis*, consistently to what already described by Sitja-Bobadilla and Alvarez-Pellittero in 1995 (Fig.3.7. G-I).

Presence of parasites in organs and different lagoons is reported in (Table.3.2.)

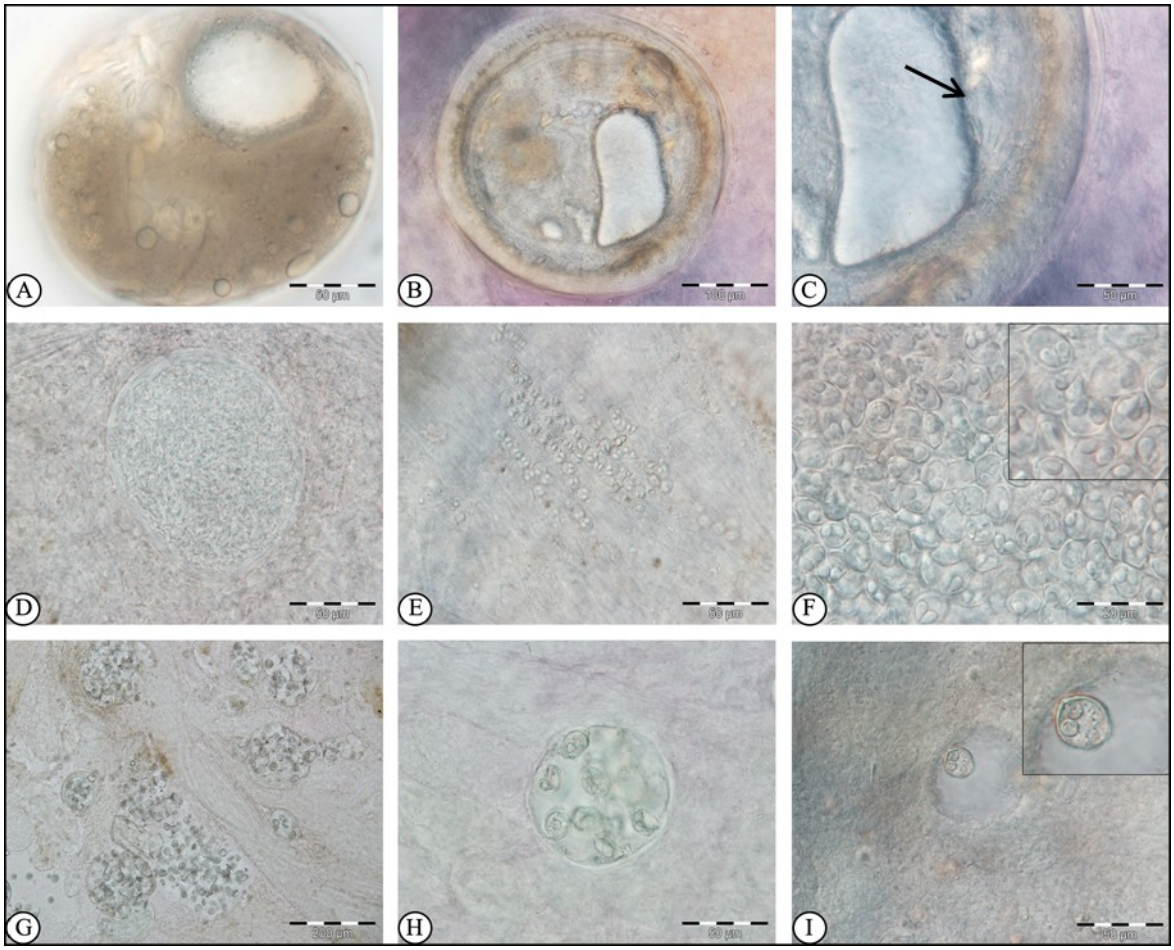

Figure 3.7. A-I. Main parasitic groups found in mullets from examined lagoons. A-C. Encysted metacercariae in heart. **A- B.** Metacercariae are folded on themselves inside cystic wall. **C**. Details of circumoral spines are visible (arrow) are used to identify genera (*Ascocotyle* sp.). **D-F.** Spores of *Myxobolus* sp. in different organs. **D.** Sporogonic cysts are faintly visualized in fresh specimens. **E.** Spores are found free in parenchyma. **F.** Sporogonic cysts. Cyst wall and polar capsules are easily identified (magnification upper right). **G-I.** *Polysporoplasma mugilis* in trunk kidney. **G.** Multifocal cysts are present in glomeruli and tubules. **H.** A single cyst contains multiple spores. **I.** Ellipsoidal shape and dimension help to identify *P.mugilis* spores (magnification in upper right).

		Heterophyes	Ascocotyle	Stictodora	Metacercariae	Myxobolus	Polysporoplasma
San Teodoro Lagoon	Heart	*	*		*	*	
	Liver				*	*	
	Spleen					*	
	Kidney					*	*
Cabras Lagoon	Heart		*			*	
	Liver					*	
	Spleen					*	
	Kidney					*	*
Calich Lagoon	Heart	*	*				
	Liver			*			
	Spleen					*	
	Kidney				*	*	*
Marceddi Lagoon	Heart						
	Liver						
	Spleen		*			*	
	Kidney						

Table 3.2. Results of parasitological examination of fresh specimens from target organs. Presence of different parasites in mullets from different lagoons. Metacercariae is referred in case of doubtful samples due to degenerative state.

3.3.3. Histopathology

Histopathologic examination revealed the presence of different lesions in all the target organs. Lesions in organs were classified in relation to fish metazoan parasites infection in stained tissue sections. (Bruno D.W. et al., 2006; Nowak B. et al, 2002)

- ✓ Digenetic metacercariae
- ✓ Myxozoan *Myxobolus* spp.;
- ✓ *Polysporoplasma mugilis*.

Lesions due to digenetic metacercariae mainly consisted of encysted parasite in the parenchyma of liver, spleen, kidney and in the myocardium or in the fibroelastic tissue of cardiac bulbus. Encysted parasites were easily identified at histopathologic examination as digenetic metacercariae for the presence of suckers, visible in may sections and digestive

system (Fig. 3.8. A-C). Number of encysted metacercariae, varied widely from one to 30 parasites per examined organ section.

Myxobolus spp. parasites were frequently formed pseudocysts (sporogonic plasmodia) within parenchyma of target organs or were found scattered within melanomacrophage centers of spleen, liver and kidney. Pseudocysts were found single or in small number in all organs examined. Furthermore, Ziehl-Neelsen staining facilitated detection of free isolated spores (Fig. 3.8.D-F).

Spores of *Polysporoplasma mugilis* were detected only in trunk kidney and were located mostly in interstitial connective tissue and in glomeruli (Fig.3.8. G-I). Number of granulomas in glomeruli containing spores was variable but always higher than 3 for each examined section.

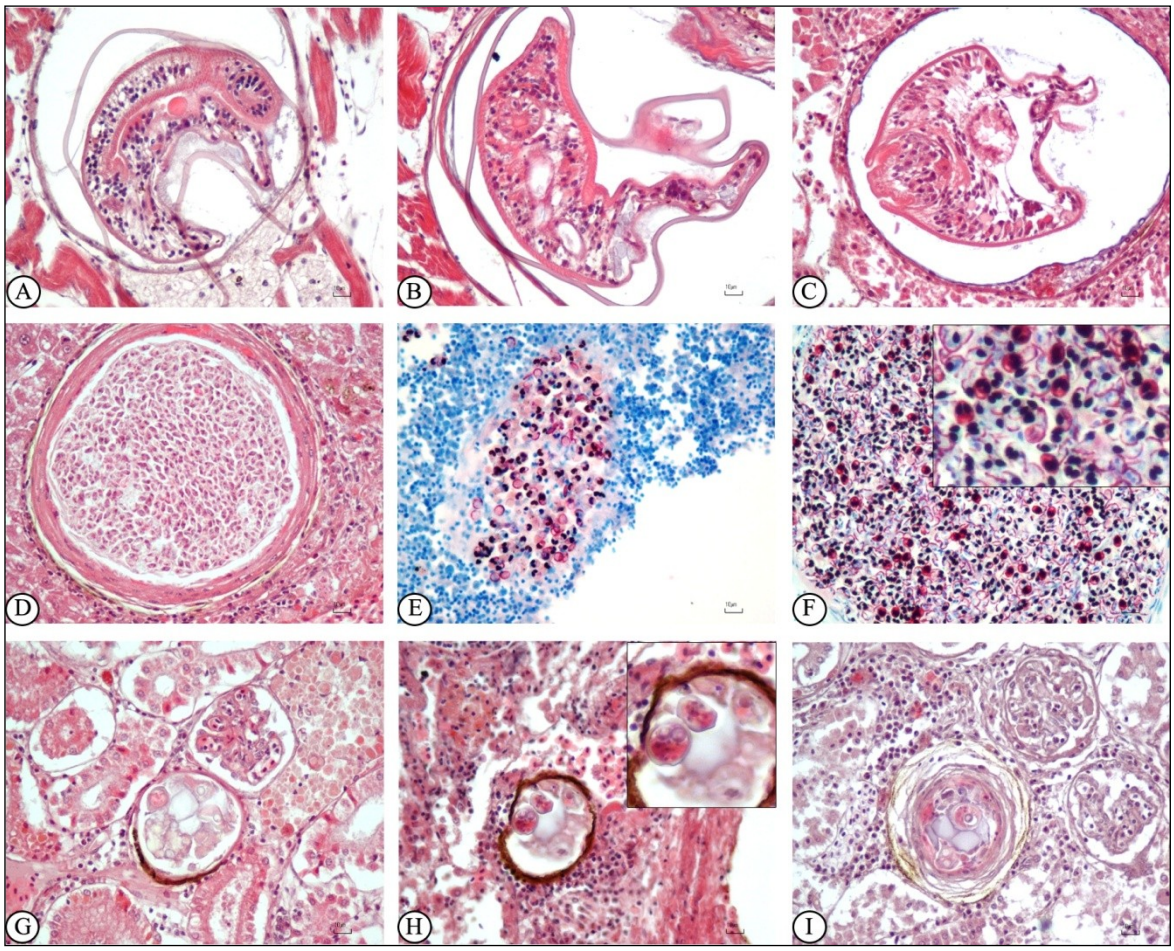

Figure 3.8. A-I. Main lesion due to different parasites of mullets from examined lagoons. A-C. Encysted metacercariae in mullet organs (HE 40X). A- B. Heart Metacercariae are folded inside the cyst. C. Kidney. Oral sucker is clearly visible (arrow). D-F. Spores of Myxobolus sp. in different organs. D. Liver. Sporogonic cysts are easily visualized in histopathologic specimens (HE). E. Spleen. Free spores are easily visualized with Ziehl-Neelsen staining (ZN 40X). F. Sporogonic cysts. Cyst wall is stained in red and polar capsules in dark blue (magnification upper right) (ZN 40X). G-I. Polysporoplasma mugilis in trunk kidney (HE 40X). G. Spores in degradation in glomeruli (HE 40X). H. *P.mugilis* spores display characteristic ellipsoid shape and prominent polar capsules (magnification upper right) (HE 40X). I. Granuloma at final stage of degradation. Some debris is detectable inside glomeruli surrounded by several layers of flattened epithelioid cells (HE 40X).

3.3.4. Data evaluation

Mugilidae species abundance and prevalence in Sardinian lagoons

Statistical analysis of species abundance in collected mullet revealed that the most frequent species observed were *Liza ramada* and *Liza aurata* (Fig.3.9.). Analysis of prevalence of various fish species in the different sites revealed that in each lagoon one species was dominant over the others. In particular, *Liza ramada* was the most represented species in Cabras and Calich lagoons both in summer and autumn, while *Liza aurata* was the most prevalent species in San Teodoro and Marceddì lagoons. Noteworthy in Cabras lagoon only *Liza ramada* species was present. Data are shown in Fig.3.9.

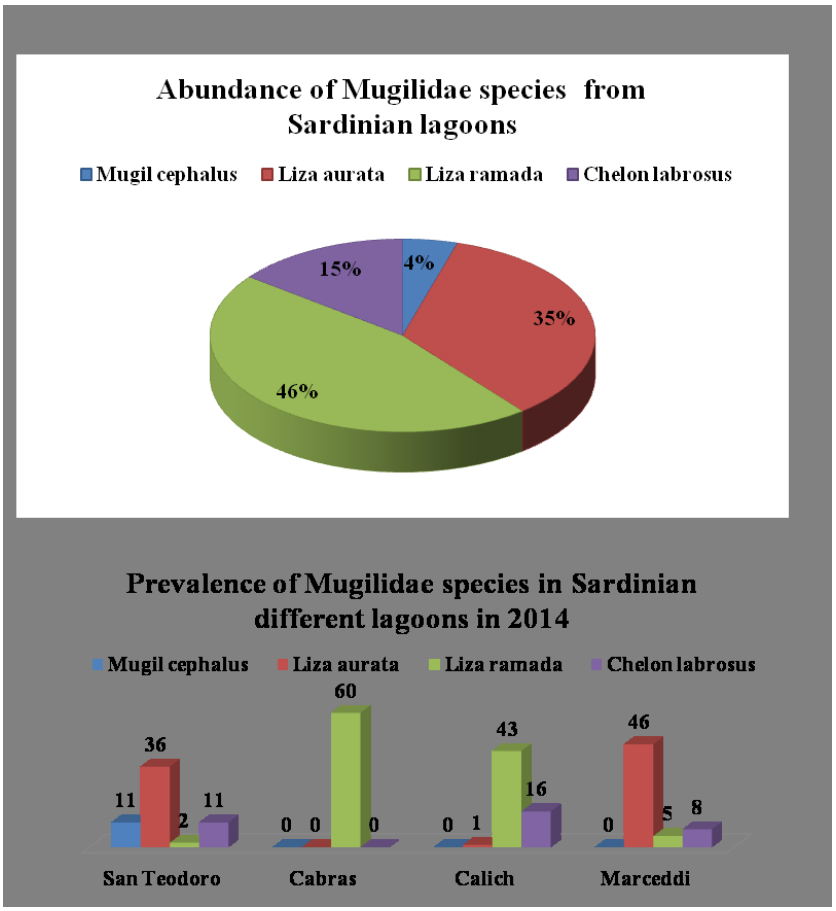

Figure 3.9. Abundance and prevalence of Mugilidae species in Sardinian lagoons. *Liza ramada* is the most represented Mugilidae species. Variety of mullet species is present in all lagoons except Cabras.

Prevalence of parasitic groups in mullets of different lagoons

Prevalence of parasitized mullets has been examined among collected fishes. Results showed that 72% of mullets (171/239) showed microscopic parasitic lesions in examined organs (liver, spleen, kidney, heart) (Fig.3.10 A). The number of infected fishes was quite constant in examined lagoons, as shown in Fig.3.10 B.

Furthermore, three different groups of parasites (Digenetic metacercariae, *Myxobolus* spp. and *Polysporoplasma mugilis*) have been found in relation with lesions observed in the organs and their prevalence has been evaluated. In particular, San Teodoro lagoon revealed to be the site with the highest prevalence of mullets [51% (27/53)] affected by digenetic metacercarie. In addition, infections caused by *Myxobolus* spp. and *P. mugilis* were also detected in 47% (25/53) and 1% of mullets, respectively (Fig.3.10. C).

Cabras lagoon showed the highest prevalence of mullets [54% (32/59)] affected by *P. mugilis*. *Myxobolus* spp. and digenetic metacercariae were also found in 32% (19/59) and in 15% (9/59) of mullets (Fig.3.10 C). In Calich lagoon ,*Myxobolus* spp was observed in 51% (31/61) of mullets, while metacercariae in 37% (23/61) and *P. mugilis* in 11% (7/61) (Fig.3.10 C)

In Marceddì lagoon, *Myxobolus* spp. affected 55% (30/55) of mullets, metacercariae 45% (25/55),whereas *P.mugilis* was never been observed (Fig.3.10 C).

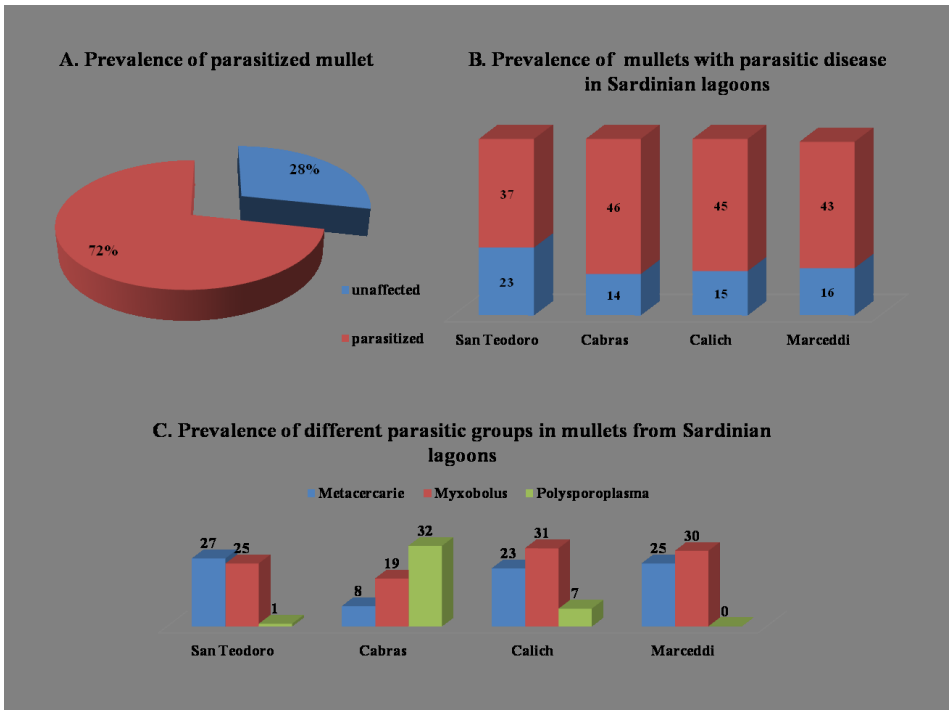

Figure 3.10. A-B. Prevalence of affected mullets among total population and in relation with different lagoons. C. Prevalence of parasitic groups in affected mullets from Sardinian lagoons.

Seasonal variability of parasites in mullets

Parasitic groups in mullets showed different values (increasing or decreasing) in the two sampling periods suggesting a seasonal variability (Fig 3.11.). These data showed that number of metacercariae and *P.mugilis* was higher in autumn than in summer, whereas Myxobolus registered a slight decrease in autumn.

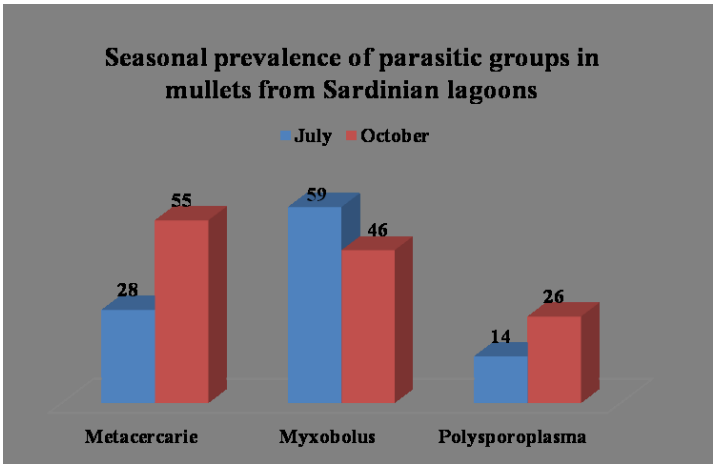

Figure 3.11. Seasonal prevalence of parasitic groups in mullets. Metacercarie and *P. mugilis*. prevalence increased in autumn whereas Myxobolus prevalence increased in summer.

Prevalence of lesions due to metacercariae in organs of mullets from sampled lagoons

Kidney and heart were the organs with higher number of lesions caused by metacercariae, representing 34% and 30%, respectively. Data are reported in Fig 3.12. A.

Different prevalence of lesions by metacercarie were observed among different lagoons in examined organs. In San Teodoro lagoon, lesions? were equally distributes in liver and spleen 43,7% (14/32). The remaining lesions were found in kidney and heart.

Cabras lagoon showed the lower prevalence of metacercariae infections 16% (10/60), distributed in all target organs with a higher prevalence in heart 8% (5/60). Calich lagoon displayed an almost equal prevalence of lesions in liver 33% (10/30), heart 30% (9/30) and kidney 26,6 % (8/30).

In Marceddì lagoon kidney showed the highest prevalence of metacercarie lesions 64,5% (20/31).

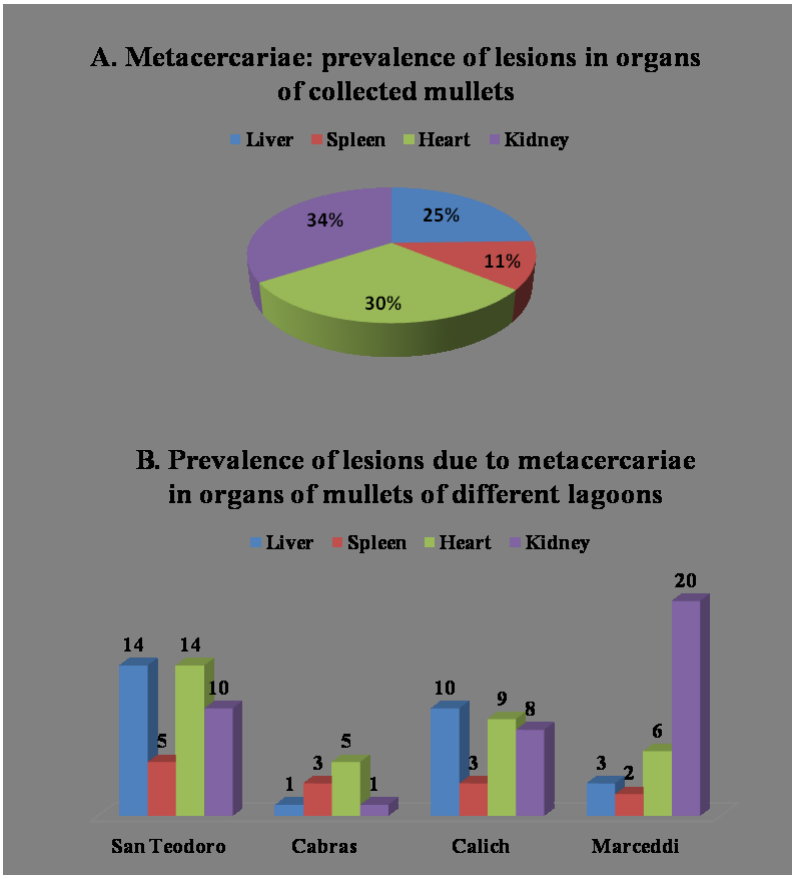

Figure 3.12. A Affected organs by metacercarie in mullets. B. Prevalence of lesions due to Digenean metacercariae.

Prevalence of lesions due to Myxobolus spp. in organs of mullets from sampled lagoons

Kidney was the most affected organ by lesions due to *Myxobolus* spp. Cabras, Calich and Marceddì lagoons showed an higher prevalence in kidney, whereas in San Teodoro lagoon lesions were mostly found in liver. Liver and spleen were almost equally affected in each lagoon. Lesions in heart were only occasionally reported (3/239 fishes) from Cabras lagoon (1/56) and Calich lagoon (2/50). Data are shown in Fig. 3.13.

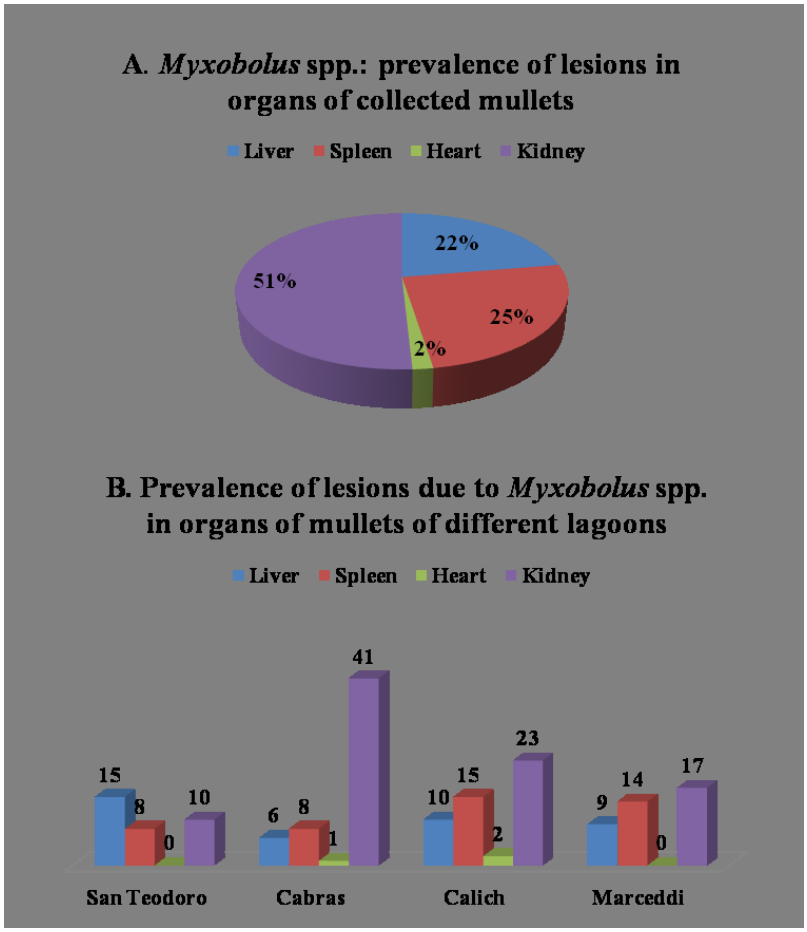

Figure 3.13. A. Kidney displayed the highest number of lesions due to Myxobolus sp. infection. B. Prevalence of lesions due to *Myxobolus* sp. in mullets. Cabras lagoon reported the highest prevalence in kidney lesions.

3.4. Discussion

Management of the complex lagoon ecosystem and the health of reared species is a challenge to maintain high productions in extensive aquaculture. In Sardinia this trade is an important economical and traditional issue and mullets represent the most important product of this farming system.

In this study Mugilidae species were collected from four different Sardinian lagoons and *Liza aurata*, *Liza ramada*, *Chelon labrosus* and *Mugil cephalus species* were included. The thirty nodular lesions grossly found in spleen, liver and heart parenchyma were related to parasites at histopathology. However microscopic examination revealed a higher number of parasitic lesions than what observed macroscopically. Parasitological examination detected the presence of digenetic metacercarie and myxosporean and permitted the identification of different parasites species in mullets affected organs. All the above mentioned species were differently present in the lagoons, except for Cabras that reported the only presence of *L.ramada.* Abundance of this species can be possibly explained because *L.ramada* prefers muddy bottoms and environments with lower salinity or freshwaters, as in Cabras lagoon, compared to other Mugilidae species (Fenza A. et al., 2014a; Almeida P.R.,1996; Lugliè A. et al., 2012). Among mullet population from different lagoons, microscopic examination revealed that 72% of fishes were affected by parasitic diseases, underlying that these form of pathologies are widely diffuse in Sardinia. In particular digenetic metacercariae were identified by parasitological examination that proved their belonging to Heterophydae family. In particular *Heterophyes* sp., *Stictodora* sp. and *Phagicola* (*Ascocotyle*) sp. were detected from fresh specimens in variable proportions in all organs in various Mugilidae species of the different lagoon. These data are aligned with what previously reported by other authors in mullets and in other fishes form Sardinia lagoons (Masala S. et al., 2014; Culurgioni J. et al., 2015). Among heterophyids *Stictodora* sp. was found only in mullets belonging to Calich lagoon, while *Phagicola* (*Ascocotyle*) was the most diffuse metacercaria in mullets of

different lagoons. These data are in accordance to what reported by other authors that found a higher prevalence of *Phagicola* (*Ascocotyle*) in mullets (Masala S. et al., 2014; Culurgioni J. et al., 2015). Culurgioni et al. reported a high prevalence of *Stictodora* sp. in Sparus aurata, while Masala et al. reported a higher prevalence in muscles of various Mugilidae species form Mistras lagoon. The low prevalence of *Stictodora* sp. observed in this study could be related to the fact that in our study some tissues, such as muscle, were not considered.

Lesions caused by metacercariae were almost equally distributed in mullet organs and percentages of organs affected by these parasites were similar. This fact could suggest a nonspecific infecting behavior of digenetic metacercariae in mullets that goes beyond differences of parasite species and different Mugilidae affected.

Also the prevalence of affected mullets by metacercarie was similar among the different lagoons. In Sardinia the abundance of Heterophyids in mullets has already been described by other authors and our data confirm what previously reported (Masala S. et al., 2014; Culurgioni J. et al., 2015). However the low prevalence of metacercarial infection observed in Cabras lagoons (13,5%) could be related to the only species collected (*L.ramada)* or could reflect a poor variety of intermediate hosts for Heterophyids in this lagoon.

Myxobolus species were found in sporogonic cysts in all examined organs. The prevalence of lesions was mainly found in kidney. Other affected organs were spleen and liver. Occasional lesions were found in the heart. Breaking down this data, in accordance to the contribution of the different lagoons, is evident that the highest prevalence of kidney lesions caused by *Myxobolus* spp. was found in Cabras lagoon, that alone represents 45% of all *Myxobolus* encysted in kidney. Because in Cabras lagoon was present only *L.ramada*, this could represent a preference of *Myxobolus* sp. for this mullet species. However, because the kidney is the most affected organ by *Myxobolus* also in Calich an Marceddì, the presence of this parasites could be related to the different lagoons environments.

The third parasitic disease examined in this study is represented by *Polysporoplasma mugilis*. It has been easily identified and differentiated from *Myxobolus* spp. in H.E. sections, but for its classification parasitological examination was determinant. It has been observed only in trunk kidney where it occupied glomeruli and interstitial connective tissue, in accordance to what reported by other authors (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995; Kent M.L. et al., 2001). Unlike the above mentioned parasites *P. mugilis* was observed only in 3 out of 4 lagoons. Cabras and Calich lagoon registered the highest prevalence of affected mullets. The absence of *P.mugilis* in Marceddì lagoon could be probably explained with the lack of intermediate hosts that don't find here a favorable environment. Mullets affected by *P.mugilis* were almost all of *L.ramada* species. However because in Cabras lagoon the only collected species was *L.ramada* it's difficult to evaluate if *P.mugilis* presence could be related to this mullet species. Our data confirm *Liza ramada* and *Chelon labrosus* as intermediate host of *P.mugilis*, as reported by previous authors (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995; Kent M.L. et al., 2001).

Moreover, seasonal prevalence of parasites it has been evaluated for the different parasitic groups. Digenean metacercariae seemed to increase in the autumn as well as *P.mugilis*, while *Myxobolus* spp. appears to follow the opposite trend.

The trend of metacercariae could be explained with temperature dependent release of cercariae from the first intermediate host and with time of fish exposure to cercariae. (Elsheikha H.M. and Elshazly A.M., 2008). Rising of water temperature during late spring and summer favorites initial infection of fishes that reach its maximum in autumn after a long period of exposure.

Myxobolus sp. showed a higher prevalence in summer season. As different *Myxobolus* species of this study has not been identified and life cycle of many Myxosporean is still not clear, it's difficult to correlate its prevalence to different variants (intermediate hosts, salinity and water temperature, etc.).

3.5. Conclusions

This study represents the first survey on the prevalence of lesions associated to parasites in mullets from different Sardinian lagoons. Histopathologic examination permitted to detect microscopic lesions in apparently unaffected organs. Results confirmed the presence of Heterophyidae trematodes in mullets from Sardinian lagoons, extending our study to different wetlands in Sardinia and in two seasons of sampling. This constitutes an important indicator of zoonotic risks from consumption of improperly prepared fillets of mullets or their derived products.

The presence of *Myxobolus* spp. still need a deeper evaluation to better characterize involved species and study their prevalence in different ecosystems.

At the best of our knowledge, this is the first description of the presence of *P.mugilis* in mullets from Sardinian lagoons.

Lagoons are complex and delicate ecosystems constituted by a net of interlacing links between parasites and their hosts. Parasites richness is a valuable indicator of a wealthy ecosystem: scarce or absent intermediate hosts could importantly affect the presence of parasites in fishes. (Elsheikha H.M. and Elshazly A.M., 2008; Culurgioni J. et al., 2015; Dzikowski R. et al., 2003). A better understanding of the dynamics in composition of parasitofauna could be an important tool to evaluate the environmental changes of Sardinian lagoons.

4. Study of the parasitic granuloma and the cellular inflammatory component

4.1. Introduction

4. 1.1. Chronic inflammatory granulomatous response in fish

Granulomatous reactions in fish have been frequently reported in association to different noxious agents, such as extraneous material, bacteria, fungi and parasites. Among these agents, parasites are the group of pathogens most widely described associated to the development of chronic granulomatous reaction (reference if possible). Many species have been reported to infect mullets and elicit a chronic inflammatory response including granuloma formation (Roberts D., 2001; Ferguson H., 2006; Noga E.J. et al., 1989; Dezfuli B.S. et al., 2013; Dezfuli B.S. et al., 2015).

Granuloma development against Digenean metacercaria

Granuloma caused by Digenean metacercariae have been reported in many fish species.

Granuloma development.

Experimental infection with metacercariae in liver of *Liza ramada* juveniles and other studies have been performed to evaluate progressive encystation of the parasite and host inflammatory response (Faliex E, 1991). Sommerville et al. described the sequence of granuloma formation in muscle and fins, that comprised the addition of the subsequent layers starting from the encysted metacercaria: epithelioid cells, fibroblasts and intermingled collagen fibers layers (Sommerville C., 1981).

Metacercaria can be found encysted after 1 day post infection (Faliex E, 1991), enveloped by a thin cyst wall, that has been described as composed of two parts. The inner layer, of mucopolysaccaride origin and produced by the parasite, is in contact with the metacercaria and the outer one, composed by flattened cells of surrounding liver parenchyma by mechanical compression in contact with unaffected organ (Faliex E, 1991; Huizinga H.W. and

Nadakavukaren M.J., 1997). Host-parasite interface results thus to be of both parasite and host origin. The presence of layers from the parasite and host tissue in the cyst structure could probably have a protective function against host immunitary response (Faliex E, 1991; Sitjà-Bobadilla A., 2008). This hypothesis is strengthened by the scarce number of inflammatory cells around encysted parasite. It has also been showed that some trematodes can expose on their surface host-like molecules able to confound immunitary system of fish (Sitjà-Bobadilla A., 2008). Two months post infection further layers of epithelioid cells (macrophages transformed in epithelioid cells and then syncytium) could be detected around encysted metacercaria. Has been proven that these cells contain filamentous filopodia and desmosomal junctions, fused to form a syncytium (Dezfuli B.S. et al., 2013; Huizinga H.W. and Nadakavukaren M.J. 1997). Four months post infection cyst wall was thicker due to fibroblasts forming several outer layers (Faliex E, 1991; Huizinga H.W. and Nadakavukaren M.J., 1997). Matrix containing eosinophils, eosinophilic granulocytes and macrophages was observed in the outer layer of fibroblasts (Dezfuli B.S. et al., 2013; Huizinga H.W. and Nadakavukaren M.J., 1997; Dezfuli B.S. et al., 2005). Dezfuli et al. in 2005 proved that this fiber network contained also nervous fibers that were immunoreactive to bombesin, serotonin-like substance, and other neuromediator molecules. This suggested that parasites could be able to create a molecular network to interfere with host immunitary response (Dezfuli B.S. et al., 2005).

In another study authors reported that several months post infection metacercariae were found dead in the hepatic parenchyma of experimentally infected *L.aurata* (Faliex E, 1991), suggesting at lifespan of the metacercariae is around eight months. Observation of metacercariae at different stage levels in the same fish or organ indicates that fish could be exposed to many infective episodes, suggesting long period of cercariae releasing from first intermediate hosts (Faliex E, 1991). Encystation has also been proven to represent an escape

mechanism from host immunitary response adopted by parasite to reach another host after fish predation (Sitjà-Bobadilla A., 2008).

Increased number of eosinophilic granular cells, neutrophils and macrophages have been reported around encysted metacercariae (Dezfuli B.S. et al., 2005; Dezfuli B.S. et al., 2015). Macrophages combined with antiserum of uninfected trout has been proved to be effective in killing 80% of metacercariae of *Diplostomules* in infected trout. As well as serum of uninfected *Chelon labrosus* was found to kill metacercariae of *Cryptocoyle lingua* in infected mullets (Dezfuli B.S, et al., 2013; Woo P.T.K., 1992). Granulocytes and macrophage were observed attached to metacercarie cyst wall 48 h post infection in *Carassius auratus* (Huizinga H.W. and Nadakavukaren M.J., 1997). These data demonstrate the active phagocytic role of macrophages in the immunitary response against metacercariae.

Eosinophilic granular cells (EGCs) have also been reported in association to other inflammatory cells around encysted metacercaria (Dezfuli B.S. et al., 2013; Huizinga H.W. and Nadakavukaren M.J., 1997; Dezfuli B.S, et al., 2008). In an experimental infection with metacercariae of lateral line scales of *Carassius auratus* , a mixed severe inflammatory infiltrate was observed, with a predominant component of EGCs (Huizinga H.W. and Nadakavukaren M.J., 1997). Authors reported that 90% of encysted metacercariae were expulsed from lateral line canal and suggested that EGCs could have an active role of by the release of substances against parasite (Huizinga H.W. and Nadakavukaren M.J., 1997). Similar observations have been made by Sommerville in 1981 in flatfish species. Studies on metacercariae infection in visceral organs or deeper tissues revealed that parasites were more likely to undergone retention than be expelled (Sommerville C., 1981; Shareef P.a.A. and Abidi S.M.A., 2012). In visceral organs, such as heart and liver, infected with digenean metacercaria, rodlet cells and EGCs were found in association with macrophages. Rodlet cells, EGCs and neutrophils were mostly observed in blood vessels surrounding the parasites while macrophages were identified around encysted metacercariae (Dezfuli B.S, et al., 2013).

Finally, rodlet cells have been frequently reported in metacercarial infection, although their role in the inflammatory response is not completely understood (Reite O.B. and Evensen, Ø., 2006; Matisz C.E., et al., 2010). Some authors hypostasized that rodlet cells could be associated to fibroblast action against parasite diffusion, rather than exert a direct action on parasite elimination. This hypothesis was explained by their scarce number in early stages of metacercariae infections, when the parasite should be more vulnerable an more easily eliminated (Matisz C.E., et al., 2010).

Granuloma due to Myxobolus spp.

Histozoic myxosporean parasites are commonly found encysted in fish tissues. They are considered more advanced in the evolutionary process than coelozoic forms (Bruno D.W., et al., 2006). Spores are generally found in fish hosts, and are visualized in HE sections. Polar capsules can be highlighted with Giemsa staining, while spores are readily visualized with Ziehl-Neelsen (Bruno D.W. et al., 2006, Nowak B. et al., 2002). Myxobolus species are reported in a high variety of fish species, both freshwater and marine, and pathogenicity can be highly variable, depending on host species and health status, site of infection and *Myxobolus* species involved (Bruno D.W. et al., 2006; Woo P.T.K., 1992). Myxozoans can also influence the inflammatory response of fishes decreasing the activity of immune cells direct against them (Sitjà-Bobadilla A., 2008). *Myxobolus* spp. in fish tissues can be found in spores but also in organized polysporic plasmodia in different tissue or single cysts in spleen, liver and kidney and cause variable degree of damage (Dykovà I. and Lom J. 2007; Molnár K. et al., 2009). Polysporic plasmodia generally elicit a variable inflammatory response that mostly lead to their delimitation by encapsulation. This process exited with the formation of granulomas, characterized by the presence of macrophages and flattened epithelioid cells disposed in a circular fashion around plasmodial cysts. An outer layer of fibroblast could be detected (Dykovà I. and Lom J. 2007; Molnár K. et al., 2009). In relation

to infected organ, fish species and *Myxobolus* pathogenicity immunitary response could also be very scarce, as reported in heart, gills, etc. (Dykovà I. and Lom J. 2007; Molnár K. et al., 2009; Azevedo R.K. et al., 2014; Ye L. et al., 2014)

Granuloma due to Polysporoplasma mugilis

Polysporoplasma mugilis has been scarcely described in literature. It has been discovered in 1995 by Sitja Bobadilla et al., in glomeruli and tubules lumina of trunk kidney in *Liza aurata* (Sitja-Bobadilla A. and Alvarez-Pellitero P., 1995). However, no histopathologic description of caused lesions has been made. A similar species, *Polysporoplasma sparis* infect seabreams and has been described only in fish reared in semi intensive system, suggesting a infection dynamics conditioned by the farming system characteristics with no apparent seasonal preferences (Palenzuela O. et al., 1999). Lesion caused by *P. sparis* were described in few reports (Palenzuela O. et al., 1999; Athanassopoulou F. et al., 2004). Spores of *P. sparis* were found in many glomeruli, inside capillary vessels. Glomeruli were significantly increased in volume and spore were intermingled to glomerular cells. In a subsequent stage of infection spores were found in higher number inside glomeruli and encapsulated by a fibroconnective belt with a scarce associated inflammatory response (Palenzuela O. et al., 1999, Athanassopoulou F. et al., 2004). In naturally infected seabream, an inflammatory response was observed mainly in a later stage, and it was mainly characterized by melanomacrophages and eosinophils. Rodlet cells were present in abundant number, creating an encircling sac around infected tubules (Palenzuela O. et al., 1999). *P. sparis* was reported to exert, as other myxozoan parasites in fishes, a lowering activity of immunitary cells in many functions (Sitjà-Bobadilla A., 2008).

4.2. Material and methods

4.2.1. Histopathologic and histochemical examination

Samples of target organs (liver, spleen, heart and kidney) were 10% formalin fixed for 48h, dehydrate with increasing concentrations of alcohol and xylene in an automatic tissue processor and, finally, paraffin embedded. Sections of 3µm were obtained with a microtome (RM2245, Leica Biosystems) and Hematoxylin-eosin(HE) stained in an automatic multistainer (ST5020, Leica Biosystems). Consecutive sections were obtained and stained with Masson's Trichrome (MT), Giemsa, and Ziehl-Neelsen (ZN). Specimens were then evaluated at light microscopy (Nikon Eclipse 80i). Histological classification of granulomas associated to the parasites was made on the basis of etiological agent involved and on the evolutive stages of the disease (Nowak et al., 2002). Microphotographs were taken and lesions were classified on the base of etiological agent. In each etiological group a progressive temporal staging has been made on the basis of histopathologic features (cellular types and quantity, presence/absence of parasite, presence/absence of necrosis, other cells associated to the lesions).

Quantitative assessment of rodlet cells and eosinophilic granular cells associated to the lesions have been performed using an open source image analysis software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Collagen evaluation and quantification in association to granulomas was measured according to a grading system score on Masson Trichrome stained sections (range 0-3: 0 = absence of collagen fibers, 1 = 1 layer of collagen fibers, 2 = 2-3 layers, 3 = ≥4 layers).

4.2.2. Immunohistochemestry

Parasitic-related granulomas were further investigate by means of immunochemical technique. In particular, additional serial sections (4 µm in thickness) were obtained from selected samples and mounted on positively charged slides (Superfrost, Fisher Scientific).

Slides were immersed for 20 min in a 98°C, preheated solution (WCAP, citrate pH 6, BiOptica, Milan, Italy) that simultaneously allows dewaxing, rehydration and antigen unmasking. Briefly, slides were mounted in a humidity chamber (Shandon, Runcorn, UK) and tissues were then blocked for endogenous peroxidase with a 15 min incubation in Dako REAL Peroxidase-Blocking Solution (S2023, Dako, Glostrup, Denmark), and for non-specific binding with 2.5% normal horse serum (ImmPRESS reagent kit,Vector Labs, Burlingame, CA, USA) for 30 minutes at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies:

- anti-cytokeratin AE1-AE3, dilution 1: 250 (Dako, Monoclonal mouse Anti-Human Cytokeratin CKAE1/AE3) to evaluate epithelioid cells in granulomas structures (Groff J.M. et al., 1997)
- anti-Vimentin, dilution 1: 500 (Dako, Monoclonal Mouse Anti-Vimentin cloneV9) to evaluate fibroblastic cells in granulomas structures (Schaffeld M. et al. 2001)

Then, sections were incubated for 40 min at room temperature with an anti-mouse secondary antibody (MP-7422, ImmPRESS reagent kit, Vector Laboratories, Burlingame, CA, USA). 3,3'-Diaminobenzidine (DAB) (ImmPACT DAB, Vector Laboratories, Burlingame, CA, USA) was used as the chromogen. All washing steps were performed three times with TBS-0.1% Tween 20 (BiOptica, Milano, Italy). Tissues were counterstained with haematoxylin, dehydrated and mounted with Eukitt® Mounting Medium (BiOptica, Milan, Italy).

Negative controls were carried out by replacing the primary antibody with PBS (Invitrogen, Milan, Italy) while fish tissues were used as positive controls.

Antibodies expression in granulomas was scored considering the number of cell layers immunostained (range 0-3: 0=absence of signal, 1=1-2 layers of immunostained cells, 2=3-5 layers, 3= ≥5 layers) by light microscopy (Nikon Eclipse 80i)

4.2.3. Statistical analysis

Macroscopical and microscopical features of the lesions were analyzed using Stata 11.2 software (StataCorp LP). Immunohistochemical results were compared using the non-parametric Kruskal-Wallis test with Dunn's *post-hoc* comparison. Furthermore, categorical and ordinal variables were compared using the Spearman rho (ρ) rank correlation coefficient. A value of ρ approximately equal to 1 indicates a good correlation, a value near 0 indicates a poor correlation, and a negative value indicates an inverse correlation. A *P*-value <0.05 was considered significant.

4.3. Results

4.3.1. Histopathologic and histochemical examination

Description of granuloma structure and staging in mugilids

Based on the cellular components, layers formation, necrosis, presence/degeneration/disappearance of parasites, the following three time-dependent stages of parasites-related granulomas have been observed in histological sections of liver, spleen, heart and kidney.

- ✓ I stage or early stage granuloma: characterized by the presence of an intact encysted parasite (metacercariae) or spore-containing cysts (*Myxobolus* spp. and *Polysporoplasma mugilis*) associated to a minimal inflammatory response (Fig.4.1.A). The observed histological features are typically found in early stage granulomas.
- ✓ II stage or intermediate stage granuloma: parasites are still visible and apparently are viable but inflammatory response increases. Inner necrotic area can be present in some cases intermingled to parasite remnants and inflammatory cells. Variable number of layers of epithelioid cells around parasites/cysts are recognizable as well as few inflammatory cells scattered around granuloma (Fig.4.1.B).
- ✓ III stage or late stage granuloma: parasites are no longer detectable inside the granulomas, substituted by a compact and intensely eosinophilic necrotic centre. Encircling layers of epithelioid cells around inner core were increased and one or more layers of fibroblasts were present. Intermingled to outer layers an increased number of inflammatory cells was observed (Fig.4.1.C).

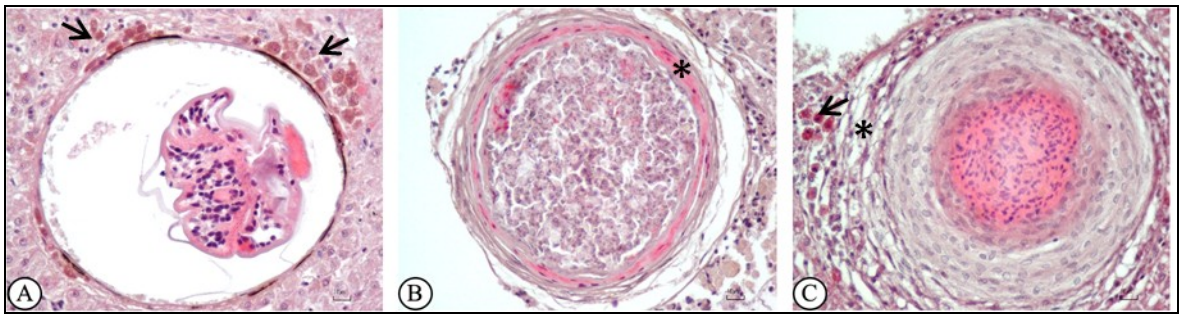

Figure 4.1.A-C. Evolutionary stages of granuloma due to metazoan parasites in mullets. A. Liver, encysted metacercaria. Early stage granuloma (I). Parasite is encysted and shows minimal inflammatory response (arrows). B. Kidney, *Myxobolus* spp. Intermediate stage (II). Epithelioid layers are evident (asterisk). C. Kidney, *Myxobolus* spp. Late stage (III). Epithelioid layers are increased and outer fibroblast layer is detectable (asterisk). Few external inflammatory cells are present (arrow).

Granuloma due to Digenean metacercaria

Granulomas associated to the presence of metacercarie were present in all examined organs. Three evolutive stages of metacercaria with different sizes, ranging from 150 to 400 µm. have been recognized.

I- Early stage: metacercariae were found encysted in parenchyma and enveloped in a thick capsule (the so-called cyst) that comprises an inner part of parasitic origin, lightly basophilic and slightly translucent, and another more eosinophilic due to the compression of host tissue cells that surrounded the cyst. Typical structures of Digenean metacercariae such as oral and ventral suckers, were recognizable and characterized by a radially arranged musculature, as well as cuticle and circumoral spines. Few inflammatory cells, mainly macrophages, were visible near the parasite or close to the cyst wall. Rodlet cells and eosinophilic granular cells have been found in a inconsistent number around encysted parasites (Fig.4.2.A).

II- Intermediate stage: the granuloma wall appeared thicker due to the increase of encircling cell layers. In particular, 1 to 3 layers of cells were clearly visible and were represented by compressed epithelioid macrophages arranged in circular whorls around the cyst wall. In few cases the presence of an outer single layer of fibroblasts, slightly compressed by granuloma

pressure and loosely in contact with the surrounding parenchyma, was observed. Macrophages or melanomacrophages (macrophages containing brown-blackish pigmentation) were increased and could be found inside the granuloma wall. Metacercaria could be observed with slight degenerative changes and scarce necrotic debris could be present. Rodlet cells were variable present, depending on organ location, while eosinophilic granular cells appeared more numerous in this stage and close to fibroblast layer (Fig.4.2.B).

III- Late stage: Metacercariae were no longer visible and the center was occupied by a intensely eosinophilic core of coagulative necrosis, intermingled to abundant degenerated melanomacrophages. Due to increased number of layers of epithelioid macrophages (>3) and fibroblast (>2) the overall structure of granuloma appears thicker compared to stage I. Furthermore, many eosinophilic granular cells were detected close to fibroblasts layer (Fig.4.2.C).

Histological features of granuloma due to Digenean metacercaria are illustrated in Fig.4.2.

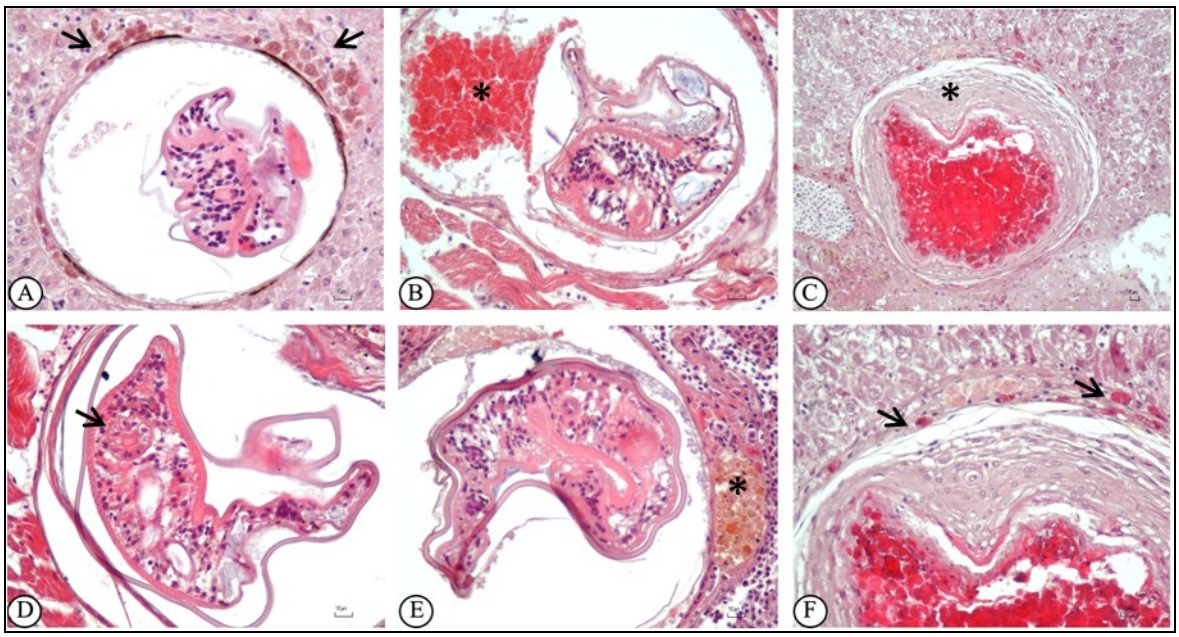

Figure 4.2. A-F. Histological features of granuloma due to digenean metacercaria in mullets. A. Liver, encysted metacercaria. Early stage granuloma (I). Scattered macrophages (arrows) around cyst wall. (HE 40X). B. Heart, encysted metacercaria. Intermediate stage (II). Necrosis (asterisk) is noticed inside parasite cyst. (HE 40X). C. Liver, encysted metacercaria. Late stage (III). Several layers of epithelioid cells encircle necrotic core are increased and outer fibroblast layer is detectable (asterisk).

(HE 20X). D. Heart, encysted metacercaria. Oral sucker (arrow) and parasite details are visible. (HE 40X). E. Spleen, encysted metacercaria. Melanomacrophages (asterisk) are in contact with cyst wall. (HE 40X). F. Liver, encysted metacercaria. Eosinophilic granular cells (arrows) are close to fibroblast layer in stage III. (HE 40X).

Granuloma due to Myxobolus spp

Lesions due to *Myxobolus* spp. were found in liver, spleen, kidney and heart. Granulomas developed a reaction to polysporic plasmodia encysted in the parenchyma. Three different stages relative to temporal progression have been observed.

I- Early stage: it was characterized by the presence of variable size (from 50 to 250 μm) polysporic plasmodia characterized by a thin eosinophilic cyst wall (of host origin) and containing a variable number (until hundreds) of *Myxobolus* spp. spores. Two to three layers of cells of surrounding parenchyma were compressed and darker due to cytoplasmic shrinkage. A scarce number of macrophages were identified near the cyst wall. Eosinophilic granular cells and rodlet cells have been observed, particularly in polysporic plasmodia localized near the vessels (Fig.4.3.A).

II- Intermediate stage: around granuloma wall a variable number of layers (1-3) of flattened epithelioid macrophages was present in addition to pre existing structures. Macrophages/melanomacrophages were often present inside granuloma space intermingled with spores. Spores were still visible and their evaluation allowed genus recognition. An outer layer of fibroblasts was variably present, generally associated to granulomas with a more degenerated content. Rodlet cells and eosinophilic granular cells number were variable (Fig.4.3.B).

III- Late stage: spores of *Myxobolus* spp. were no longer identified in this stage. Inner core was constituted by coagulative necrosis intermingled with an elevated number of melanomacrophages, with brownish to intensely eosinophilic cytoplasm, in relation to the phagocytized material. More than 3 layers of flattened epithelioid macrophages were detected

and further outer layers of fibroblasts were often observed. Eosinophilic granular cells were present in elevated number around granuloma edges or interposed to fibroblasts in outer layers (Fig.4.3.C).

Histological features of granuloma due to Myxobolus spp are illustrated in Fig.4.3.

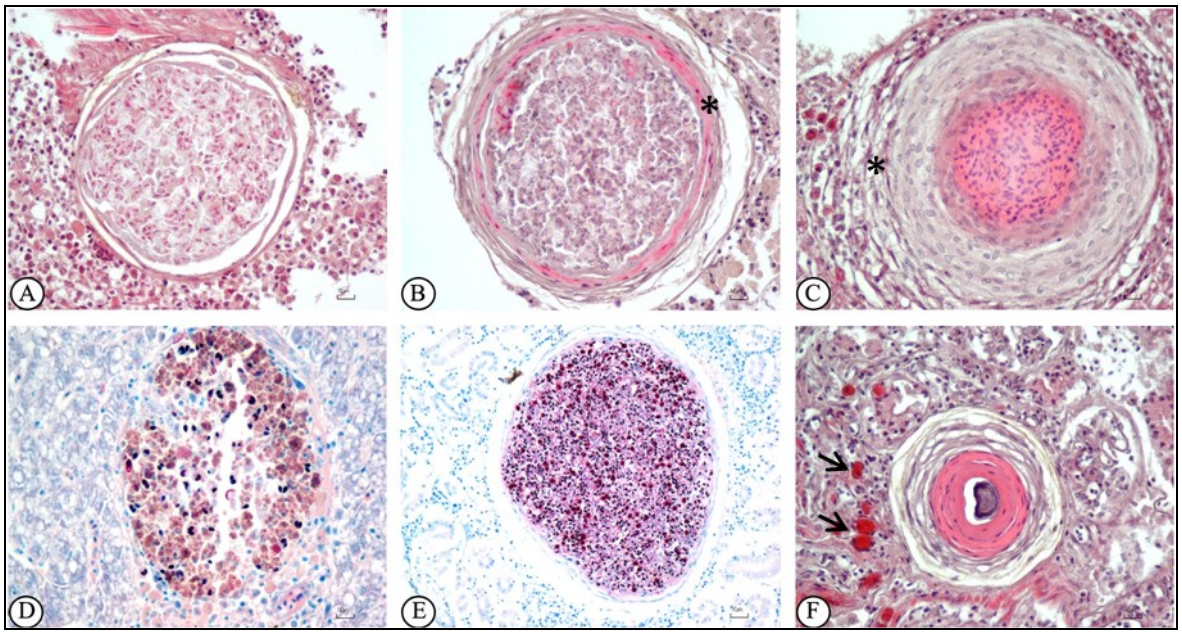

Figure 4.3. A-F. Histological features of granuloma due to *Myxobolus* spp. in mullets.

A. Spleen, polysporic plasmodia. Early stage granuloma (I). (HE 40X). B. Liver, polysporic plasmodia. Intermediate stage (II). Few layers of epithelioid cells (asterisk) are detectable. (HE 40X). C. Liver, polysporic plasmodia. Late stage granuloma (III). Several layers of fibroblasts (asterisk) are present in outer portion of granuloma. (HE 20X). D. Liver, melanomacrophages center. Spores are recognizable and stained in red and polar capsules in blue (ZN 20X). E. Kidney, polysporic plasmodia. Mature spores are highlighted by Ziehl-Neelsen staining. (ZN 20X). F. Kidney, polysporic plasmodia. Eosinophilic granular cells (arrows) are close to fibroblast layer in stage III. (HE 40X).

Granuloma due to Polysporoplasma mugilis

Lesions caused by *Polysporoplasma mugilis* were found only in trunk kidney, inside glomerular structures, or in interstitial connective tissue. Three developmental stages were recognized for *P.mugilis.*

I- Early stage: lesions caused by spores, ranging from 100 to 200 µm, were characterized by the presence of a inner slightly eosinophilic wall, constituted by compressed glomeruli elements (still recognizable) and an outer thin layer attributable to the basal membranes of glomeruli . Each lesions contained a variable number (tens to hundreds) of *P.mugilis.* spores. A scarce number of macrophages was identify in the vicinity of affected glomeruli/tubules, as well as eosinophilic granular cells and rodlet cells (Fig.4.4.A).

II- Intermediate stage: spores were still visible inside dilated glomerular spaces, intermingled to macrophages. Necrotic debris and degenerate spores were present. One to three layers of epithelioid cells were detected around infected glomerular+structures, and an outer layer of fibroblasts was observed. Eosinophilic granular cells and rodlet cells were variably present (Fig.4.4.B).

III- Late stage: spores were no longer detectable inside granulomas, substituted by a compact core of coagulative necrosis. Flattened epithelioid macrophages were organized in more than 3 layers and further layers of fibroblasts were present in the outer portion of granulomas. Eosinophilic granular cells and rodlet cells were found in an elevated number around granulomas near fibroblast layers (Fig.4.4.C).

Histological features of granuloma due to *Polysporoplasma mugilis* are illustrated in Fig.4.2.

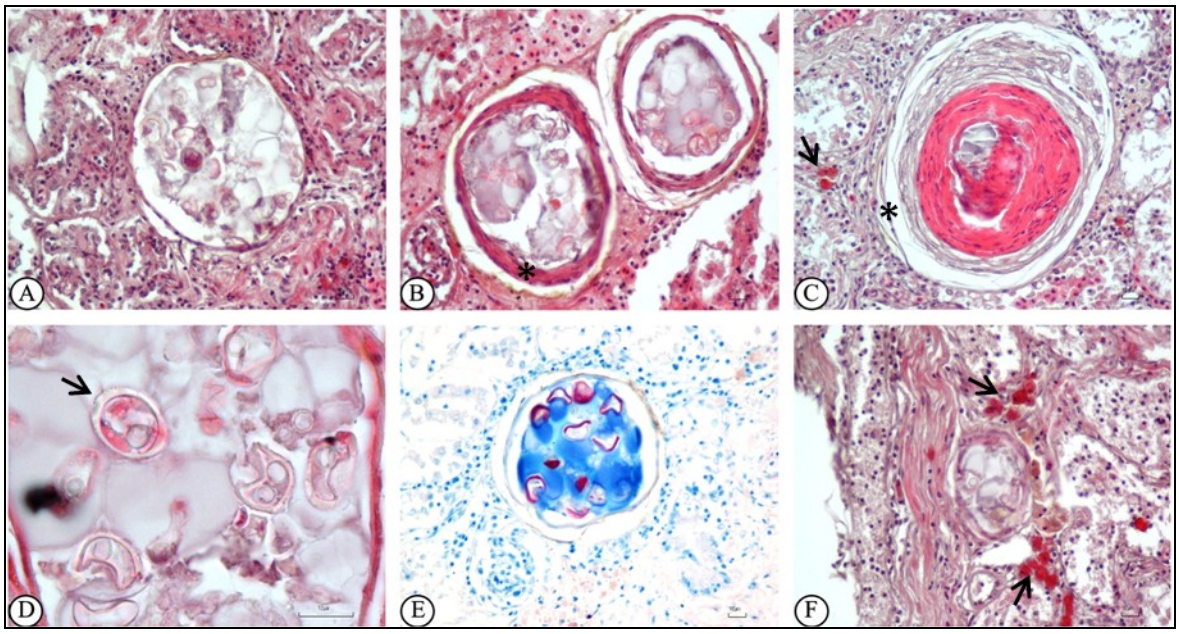

Figure 4.4.A-F. Histological features of granuloma due to *Polysporoplasma mugilis* in mullets.
A-F. Kidney. Spores in glomerular spaces. A. Early stage granuloma (I). (HE 40X). B. Intermediate stage (II). Few layers of epithelioid cells (asterisk) are detectable (HE 40X). C. Late stage (III) Eosinophilic granular cells (arrow) are near fibroblasts layers (asterisk) (HE 20X). Spores are still recognizable inside glomerular spaces. (HE 100X OIL). E. Spores are stained in red by Ziehl-Neelsen staining. (ZN 20X). F. Eosinophilic granular cells (arrows) are close to granuloma . (HE 40X).

Identification and evaluation of rodlet cells

Rodlet cells has been observed in HE sections of liver, spleen, heart and kidney, located in vascular walls, bile ducts, and periportal spaces both in unaffected and parasitized organs (Fig. 4.5). Furthermore, these cells were also particularly found associated to the parasitic granulomas, as further demonstrate by the significant positive correlation between the presence of rodlet cells and granulomas due to *Myxobolus* spp. ($\rho = 0.4296$, $P < 0.05$). Moreover, no statistical significant difference were observed between the number of the cells and the different stages of granulomas (Kruskal-Wallis χ^2 (corrected for ties) =5.940, P = 0.051).

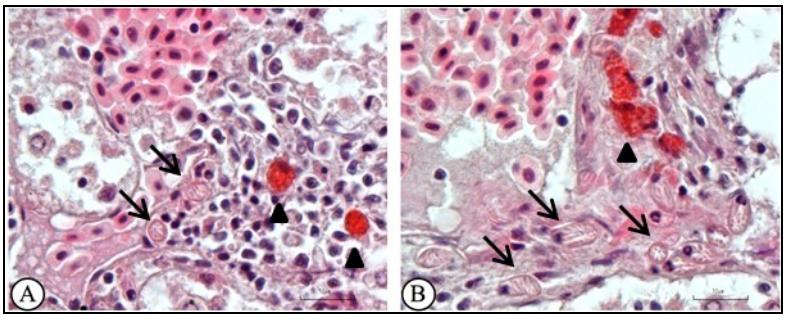

Figure 4.5. Rodlet cells in tissues of mullets. Kidney. Rodlet cells (arrows) are more abundant close to vessels and eosinophilic granular cells (arrowhead). (HE40X).

Identification of collagen component

Collagen component was evaluated in sections stained with Masson trichrome (bright blue). It was found in each organ as a component of vascular structure, basal membranes and glomerular structures (Fig. 4.6.A-C). Furthermore collagen component was a prominent features of intermediate and late stage granulomas (Kruskal-Wallis $\chi 2$ (corrected for ties) = 40.949, P = 0.00010) if compared to early stages (Dunn's post-hoc test, early *vs* late P= 0.00000; early *vs* intermediate P= 0.001485).

In addition, a significant positive correlation between collagen fibers and the number of eosinophilic granular cells was observed ($\rho = 0.4707$, $P < 0.05$).

Identification and evaluation of eosinophilic granular cells

Eosinophilic granular cells (EGCs) were normally found in sections stained with Giemsa in bright pink color in liver, spleen, kidney and heart near major blood vascular structures and in periportal spaces (Fig. 4.6.D-F). In addition, EGCs were also observed in association to granulomas of different parasitic groups and a significant positive correlation between the presence of these cells and granulomas caused by Digenean metacercariae was observed ($\rho = 0.5197$, $P < 0.05$). In addition, a significant association were observed between the number of cells and the different stages of granulomas (Kruskal-Wallis $\chi 2$ (corrected for ties)

=12.110, P = 0.00235). In particular a higher number of EGCs was observed in late stage granulomas compared to early ones (Dunn's *post-hoc* test, P= 0.000324).

Figure 4.6.A-F. Histological evaluation of collagen component (A-C) and eosinophilic granular cells (D-F)

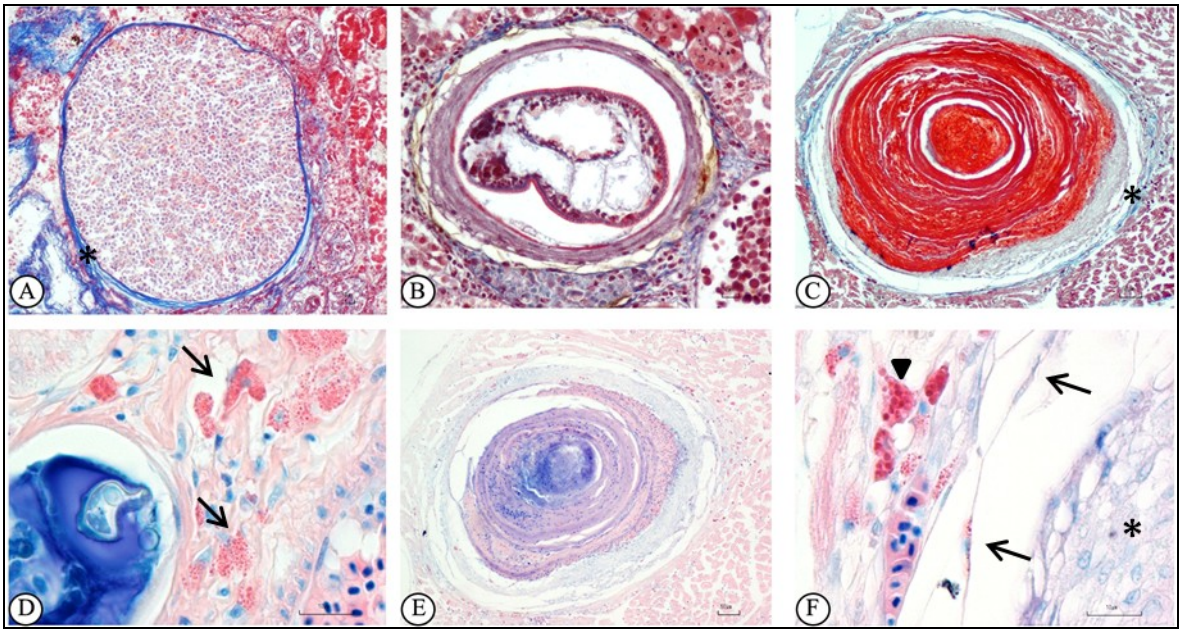

Figure 4.6.A-F. Histological evaluation of collagen component and eosinophilic granular cells.
A. Kidney. Polysporic plasmodia of *Myxobolus* spp. in granuloma stage I is encysted by basal membrane (asterisk) (MT 20X). B. Kidney, encysted metacercaria in granuloma intermediate stage (II). Few layers of collagen stained in blue are detectable (MT 40X). C. Heart, *Myxobolus* spp. in late granuloma stage (III). A thick layer of collagen (asterisk) is visible (MT 40X). D. Kidney, *Polysporoplasma mugilis*, granuloma stage II. Many eosinophilic granular cells (arrows) are close to parasites. (Giemsa 100X OIL). E. Heart. *Myxobolus* spp. in granuloma late stage (III). Scattered eosinophilic granular cells around a granuloma. (Giemsa 10X). F. High magnification of picture E (arrows). Eosinophilic granular cells (triangle) are in close contact to fibroblasts (arrows). Epithelioid layer are visible (asterisk). (Giemsa 100XOIL)

4.3.2. Immunohistochemestry

Study of phenotypic characteristics of granuloma

Identification and evaluation of epithelioid cells

Epithelioid cells were identified by a positive membranous to cytoplasmic brownish staining to anti-cytokeratin CKAE1-AE antibody. Epithelioid cells were rarely observed in granulomas stage I, but were consistently found and organized in circular layers around degenerated parasites in stage II or around necrotic core in stage III (Kruskal-Wallis $\chi 2$ (corrected for ties)= 69.254, P = 0.00010; Dunn's post-hoc test, early *vs* intermediate *vs* late P= 0.00000; intermediate *vs* late P= 0.003448). In conclusion the layers number of epithelioid cells increased with granuloma stages progression (Fig.4.7.).

Identification and evaluation of fibroblasts

Fibroblasts were detected by a positive labeling to anti-Vimentin antibody, with a strong and diffuse signal in membrane and cytoplasm. They were observed in outer portion of granulomas in contact to surrounding parenchyma. The number of fibroblast layers was increase with granuloma stages, as further demonstrated by the statistical analysis (Kruskal-Wallis $\chi 2$ (corrected for ties)= 52.233, P = 0.00010; Dunn's post-hoc test, early *vs* intermediate P= 0.000517; early *vs* late P= 0.000000, intermediate *vs* late P= 0.001709). (Fig.4.7.).

Figure 4.7.A-R. Immunohistochemestry evaluation of epithelioid cells and fibroblast related to parasite species and granuloma stages in mullets.

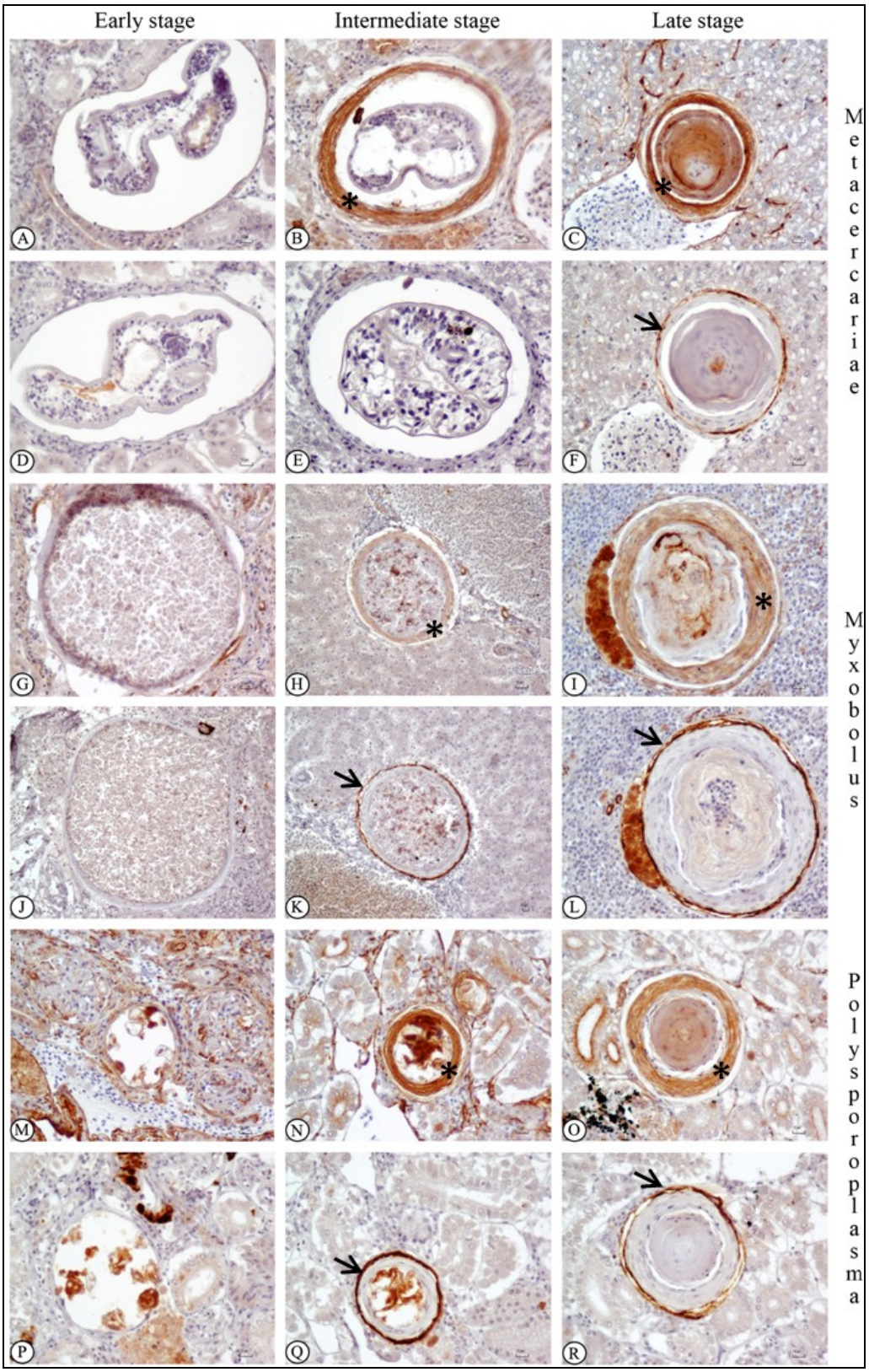

Figure 4.7.A-R. Immunohistochemestry evaluation of epithelioid cells and fibroblast.

A-F. Granulomas due to metacercarie. A-C: stained with anti CKAE1/AE3 antibody. D-F: stained with anti Vimentin antibody.

G-L. Granulomas due to *myxobolus* spp. G-I: stained with anti CKAE1/AE3 antibody. J-L: stained with anti Vimentin antibody.

M-R. Granulomas due to *Polysporoplasma mugilis.* M-O: stained with anti CKAE1/AE3 antibody.

P-R: stained with anti Vimentin antibody.

Epithelioid cells (asterisk) are identified by a moderate to strong cytoplasmic signal (brown color) in granuloma stages II and III. Fibroblasts (arrow) are identified by a strong cytoplasmic signal (brown color) in granuloma stages II and III.

4.4. Discussion

Parasitic diseases may be a relevant threat to the health of Teleostean fish and constitute a challenge to their immune system of fish due to the ability of the parasites to become cryptic to the fish response. In last few years many studies deepened the knowledge of the dynamics of the fish response against parasites and the role exerted by different components of immunitary system.

Digenean metacercariae have been found encysted in many intermediate host fish species, and particularly mullets have been reported to potentially harbor different levels of many Digenean species, particularly belonging to Heterophyidae family in the case of the Mediterranean area (Roberts D. 2001, Masala S. et al., 2014; Culurgioni J. et al., 2015).

Granulomas associated to metacercariae were found in examined visceral organs (liver, spleen, heart and kidney). They were characterized by encysted metacercariae encircled in layers of epithelioid cells and fibroblasts. This granuloma structure was commonly reported in metacercariae infestation in visceral organs (Ferguson H., 2006; Dezfuli B.S, et al., 2013). Metacercariae granulomas are also reported in association to eosinophilic granular cells (EGCs) (Reite O.B. and Evensen, Ø., 2006, Dezfuli B.S, et al., 2013). Specimens of mullets of the present study revealed a particularly high number of EGCs associated to metacercariae lesions, mainly observed in outer layers of granulomas. This observation was confirmed by a significant correlation between the number of EGCs and metacercariae infection respect to other parasitic groups ($\rho = 0.5197$, $P < 0.05$).

Myxobolus spp. are reported to infect a wide range of organs in fishes and cause variable degree of damage (Dykovà I. and Lom J. 2007; Molnár K. et al., 2009, Azevedo R.K. et al., 2014; Ye, L. et al., 2014; Madhavan R. et al., 2013). In the present study, examined organs infected with polysporic cysts didn't reveal a particular damage in surrounding tissue. Granulomas containing mature spores (the only type observed) revealed that spores of *Myxobolus* spp. were apparently delimited by a layer of host origin, that was positive to

Masson's trichrome and presumably attributable to basal membranes or vascular endothelia of the different affected organs. This is accordance of what observed by Molnar in a diffuse *Myxobolus* spp. infection (Molnár K. et al., 2009). Different developmental stages of inflammatory reaction against *Myxobolus* cysts have been observed. In literature a variable degree of immunitary response has been observed, depending on location, fish species and *Myxobolus* species pathogenicity (Dykovà I. and Lom J. 2007; Molnár K. et al., 2009; Azevedo R.K. et al., 2014; Ye L. et al., 2014).

Granulomas due to *Polysporoplasma mugilis* has been observed to infect trunk kidney of mullets of the present study. Location of spores in Bowman's spaces was confirmed by the positive staining to Masson's trichrome and anti-cytokeratin antibody that highlighted basal membrane and glomerular structures, respectively, in accordance to what reported by Gross et al., 1997 (Groff J.M. et al., 1997). Histopathologic examination of kidneys affected by *P.mugilis* revealed a moderate to severe tissue damage, due to the invasion of glomerular structures by spores, presumably leading to a partial functional loss of kidney tissue. Pathogenicity of *P.mugilis* seemed thus to be higher than that exerted by metacercariae and *Myxobolus* spp, that generally were found encysted in interstitial connective tissue or in the vicinity of blood vessels and do not excessively alter functional structures of trunk kidney. These data could not be compared to literature as, to the best of our knowledge, histopathologic features of lesions due to *P.mugilis* have not been reported yet. However, diffuse and severe glomerulonephritis has been described in seabream affected by another species belonging to Polysporoplasmidae family, *Polysporoplasma sparis,* that infected and causes severe glomerular damage (Palenzuela O. et al., 1999, Athanassopoulou F. et al., 2004). Authors reported that Bowman's capsule was thicker compared to neighboring unaffected glomeruli, probably as a consequence of parasite maturation. These features, as well as layers of epithelioid cells and periglomerular fibrosis associated to granulomas, were reported in *P.sparis* infections and in our study were observed in kidney of mullets. Degree of

damage in mullets appeared however less severe and diffuse than what reported in sea bream. Further studies are necessary to investigate *P.mugilis* pathogenesis and evaluate the resistance of mullets and the pathogenetic potential of this parasite.

Evaluation of progression of inflammatory response against the different parasitic groups lead to identification of three major granuloma developmental stages. Common histopathologic features have been observed in granulomas at the same developmental stage in all the three parasitic groups studied.

In the early stage (stage I) encysted parasites are associated to an apparently scarce or absent inflammatory reaction. A layer constituted by host compressed cells of surrounding tissue was easily recognizable in liver and heart, while in spleen and kidney this phenomena was less evident. Thus, was probably due to the looser parenchyma architecture that allowed a better settling of parasites, as suggested by other authors (Faliex E, 1991). In this stage parasite were probably hidden from immunitary system by a "protective coat" provided by host tissue that is recognized as "self" from immunitary cells. This is a recognized escape mechanism of parasites from host immunitary response (Sitjà-Bobadilla A., 2008). Moreover, the scarce inflammatory response elicited in this stage could be explained by a regulatory action that Digenean metacercariae and myxozoan are able to exert to lower the activity of immunitary cells (Sitjà-Bobadilla A., 2008).

Granuloma Stage II (intermediate) represents the first evidence of fish immunitary response directed toward isolation of parasites. Hallmark of this stage is the presence of epithelioid macrophages that encircles parasites and avoid its diffusion in surrounding parenchyma (Ferguson H., 2006; Noga E.J. et al., 1989; López-Dóriga M.V. and Martínez J.L. 1998) The increase of layers of epithelioid cells during granulomas developement showedstatistically significant association (Kruskal-Wallis $\chi 2$ (corrected for ties)= 69.254, P = 0.00010; Dunn's post-hoc test, early *vs* intermediate *vs* late P= 0.00000; intermediate *vs* late P= 0.003448), and represented an easily detectable feature to granuloma stage identification. These cells were

particularly discernible with IHC staining, as they showed a strong positive labeling to anti-cytokeratin antibody (CKAE1/AE3) in accordance to what reported by other authors in different fish species (Noga E.J. et al., 1989; Groff J.M. et al., 1997).

Late granuloma stage (III) represents the resolution of parasite infection. Fibroblasts have proven a useful tool for stage recognition, as they increased with progression of evolutive stages, reaching their maximum expression in III granuloma stage. This observation has been confirmed by statistically significant correlation between vimentin expression and different granuloma stages (Kruskal-Wallis $\chi 2$ (corrected for ties)= 52.233, P = 0.00010; Dunn's post-hoc test, early *vs* intermediate P= 0.000517; early *vs* late P= 0.000000, intermediate *vs* late P= 0.001709). Vimentin was indeed used to identify fibroblasts associated to granulomas. Only few studies investigated the expression of vimentin in teleostean fish tissues (Schaffeld M. et al. 2001; Herrmann H., 1996). According to what reported by these authors, fibroblast should have shown an intense label with anti-cytokeratin antibody and lack of signal with anti-vimentin antibody (Groff J.M. et al., 1997; Schaffeld M. et al. 2001). Our investigation showed an opposite result, as vimentin positive cells were identified in the present study as fibroblasts. Serial sections of granulomas stained with anti-cytokeratin and anti-vimentin antibodies, clearly highlighted that areas occupied by epithelioid cells (CKAE1/AE3+) and by putative fibroblasts (VIM+) were not overlapping, making unlikely a misunderstanding the different origin of these cells. Possible reasons of differences in expression of vimentin and cytokeratin molecules, could be attributed to the fact that teleostean represent a wide animal group that comprises species phylogenetically distant to each other, as fish species of previous studies (trout and shark).

Histological features correlated to granulomas in different evolutive stages have been already investigated by many authors, in experimental infections with various parasites or injection of foreign material (Timur M. et al., 1977; López-Dóriga M.V. and Martínez, J.L. 1998; Dezfuli

B.S. et al., 2013). However, to the best of our knowledge, a histopathologic study on response to different parasites among the same host population has not been performed.

Granulomas in different evolutive stages were observed in the same specimen. This could be related to different episodes of infections in the same mullet, in accordance to what already suggested by other authors (Faliex E, 1991).

Granulomas structure investigated in the present study presents similar features in different parasitic groups, suggesting that immunitary system in mullets displays a common response to metazoan parasites infection in internal organs.

The immunitary response to metazoan parasites in Mugilidae species seems therefore to be not specifically adapted to a class of parasites, but nonspecific and mainly characterized by encapsulation mechanism, at least in visceral organs. Encapsulation is also considered as one of the most common mechanism exerted by parasites to escape from host immunitary response (Sitjà-Bobadilla A., 2008).

Isolation of parasites is an articulated mechanism that consists of the action of different immunitary cells, such as macrophages, epithelioid cells, eosinophilic granular cells, rodlet cells and also connective elements, such as fibroblasts.

Macrophages (MAs) were detected scattered around granulomas in stage I in each investigated parasitic groups and thus considered the first immunitary cell to approach parasites. This is in accordance to what reported in early parasites infections in fishes, that describe these cells in the forefront of immunitary response (Roberts D. 2001; Ferguson H., 2006; Woo P.T.K., 1992). Macrophages are also considered the most important phagocytes in teleosts, as they provide to destroy and eliminate noxious agent (Agius C. and Roberts R.J. 2003; Secombes C., 1999; Dezfuli B.S. et al., 2013). Our observations in granulomas of II and III stage support this theory, as MAs were observed in direct contact to parasites remnants or engulfed with necrotic material.

Encircling and sequestration of parasites has been reported as a simple but effective system to reduce parasite diffusion (Ferguson H., 2006,Noga E.J. et al., 1989). Epithelioid cells exert a decisive role in delimiting parasite in parenchyma, and this has been extensively reported in literature (Ferguson H., 2006; Noga E.J. et al., 1989; Dezfuli B.S. et al., 2000; López-Dóriga M.V. and Martínez J.L. 1998; Dezfuli B.S. et al., 2013). They are described to form a corona of several layers around encysted parasites and has have been observed in granulomas of examined parasitic groups.

During inflammatory response against parasites, epithelioid cells are described like a corona of one or more layers of flattened macrophages that tightly surround parasite. At ultrastructural examination the inner layer of epithelioid cells shows finger-like projections (filopodia) that adhere to parasites and penetrate its cuticle. The cell contains many filaments, mitochondria and euchromatic nucleus (Dezfuli, B.S.et al., 2000). In case of persistent antigen macrophages can differentiate in epithelioid cells that surround the pathogenic material and isolate it from parenchyma (Noga E.J. et al., 1989). Encystation process is probably a primitive and nonspecific but effective mechanism for a quickly isolation of pathogens developed in vertebrates, in which desmosomes of epithelioid cells assure a secure barrier and facilitate sequestration of agents (Noga E.J. et al., 1989).

Fibroblasts have been frequently reported in association to granulomas formation in the course of parasite infections and, although they are not proper immunitary cells, are considered an integrative component of immunitary response (Roberts D. 2001, Skorobrechova E.M. and Nikishin V.P. 2011; Di Maio A. and Mladineo I. 2008). This fact has been confirmed in the present study, as fibroblasts exerted a fundamental role in delimitation of granuloma expansion with an increasing production of collagen in intermediate and late stage granulomas. A strong statistical correlation between fibroblasts and collagen fibers in granuloma stages II e III was indeed observed (Kruskal-Wallis $\chi 2$ (corrected for ties) = 40.949, P = 0.00010).

Investigation on collagen expression in granulomas in association to other inflammatory elements demonstrated a significant correlation with collagen fibers and the number of EGCs in late stage granuloma ($\rho = 0.4707$, $P < 0.05$). At microscopic examination granulomas in stage III revealed a tightly contact of eosinophilic granular cells and fibroblasts in the outer layers in accordance to what reported by other authors (Dezfuli B.S. et al., 2013; Huizinga H.W. and Nadakavukaren M.J., 1997; Dezfuli B.S. et al., 2005).

Number of EGCs have been observed to increase with granuloma development in each parasitic groups of the present study and this was confirmed by a statistically significant association (Dunn's *post-hoc* test, P= 0.000324). The role of eosinophilic granular cells in the immunitary response against parasites has been widely investigated, and different functions have been attributed to these cells. EGCs have been reported to act in early stages infection to favor parasites expulsion from fish body, by release of certain substances (Sommerville C., 1981; Dezfuli B.S.et al., 2000). Evidences produced by the present study cannot support this theory, as in doing so EGCs should have been found in higher number in early stage (I). Our data are more in agreement with what observed by other authors, that found an increased number of these cells in later stages and in association with fibroblast layers. In accordance with this theory eosinophilic granular cells could act to regulate fibroblast action in a later stage of granuloma, as postulated by many authors (Dezfuli B.S.et al., 2000). However, based on data obtained in mullets, the number of EGCs in granuloma stage III was not correlated with fibroblast presence, but with increasing collagen fibers. This result suggests that putative action exerted by EGCs could be more related to regulation of collagen production than to recruitment of fibroblasts in the site of infection.

Eosinophilic granular cells are considered precursors of mammalian mast cells (Ferguson H., 2006, Reite O.B. and Evensen Ø., 2006; Manera M. and Dezfuli B.S., 2004). An active role of mast cells in fibrotic processes has been observed in humans, in association to different

diseases and parasites, characterized by induction of collagen production under secretion of profibrotic molecules (Hügle T., 2014; Andrade Z.A., 2009).

Really, has been postulated that mast cells can probably exert a modulation of their function in relation to the site of infection. In organs with an open route toward outside (lungs or intestine) mast cells act to eliminate the parasite, while in visceral organs they promote its encapsulation by an alternative pathway (Hügle T., 2014). A similar mechanism has been hypothesized for EGCs in course of parasitic diseases in fish (Huizinga H.W. and Nadakavukaren M.J., 1997).

Rodlet cells (RCs) still represent an enigma in Teleostean pathology. Although widely investigated, their role appears not completely elucidated. Some authors suggested a down regulatory action of RCs on epithelioid cells in late stage of inflammatory response against parasites (Dezfuli B.S.et al., 2000), while in infections of gills or intestine they have been reported to exert a direct and early action to favor parasite detachment and elimination (Manera M. and Dezfuli B.S., 2004; Dezfuli B.S.et al., 2007). The present study revealed their presence in variable number in each stage of granulomas but lacked to find a correlation between their number and progressive granuolma developmental stages. A significantly statistical association ($\rho = 0.4296$, $P < 0.05$) has been instead observed between the number of RCs and lesions due to *Myxobolus* spp. Increase of these cells in Myxozoan infections has been frequently reported (Dykovà I. and Lom J. 2007; Ferguson H., 2006) but to our knowledge no comparative study on prevalence of RCs associated to different parasitic groups in the same fish population have been reported.

4.5. Conclusion

The present study highlighted that immunitary system in mullets displays a common response to metazoan parasites infection in internal organs. The immunitary response to metazoan parasites in Mugilidae species seems therefore nonspecific and mainly characterized by encapsulation mechanism.

To the best of our knowledge this study represents the first description of histopathologic and immunohistochemical features of lesions caused by *P.mugilis* in kidney of Mugilidae species. Pathogenicity of this parasite seemed higher than that exerted by metacercariae and *Myxobolus* spp.

Isolation of parasites is an articulated mechanism that consists of the action of different immunitary cells, such as macrophages, epithelioid cells, eosinophilic granular cells, rodlet cells and also connective elements, such as fibroblasts.

Further studies are needed to elucidate host-parasite interaction and the role of different cellular components involved in fish inflammatory response

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Acknowledgements

I would like to show my gratitude to Dr. Elisabetta Antuofermo, for being a guide and for being a constant source of motivation for improvement in this wonderful work.

I would like to thank Dr. Francesc Padros for his help in scientific "matter" and for showing me an enthusiastic approach to aquatic world.

I also would like to thank Dr. Paolo Merella for patiently attending the parasitological examination and Dr Antonio Pais for his precious contribution to development of aquaculture section.

I would also like to thank all component of Anatomic Pathology section: Dr. Marina Sanna for her irreplaceable technical support, Dr. Roberta Ariu and Dr. Giovanni Burrai for their willingness and their help in different phases of this work, and all the colleagues for being more than colleagues in these years.

A special thanks is dedicated to my husband Giampaolo for his patience and for his support during these years.

Last but not least I want to thanks my parents and my "larger" family for giving me the opportunity to reach this goal in many different ways.