UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO DI RICERCA
Scienze e Biotecnologie
dei Sistemi Agrari e Forestali
e delle Produzioni Alimentari

Indirizzo Scienze e Tecnologie Zootecniche

Ciclo XXVIII

Influence of pathogens and pollution on Mugilidae health: first evidence of mycobacteriosis and intersex condition in extensively reared mullets from Sardinian lagoons

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Anno accademico 2014-2015

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Abstract

The multifactorial nature of fish health and diseases are generally linked to an imbalance of pathogen, resistance of the fish and environmental stress. Pollution is considered an anthropogenic factor that can affect fish life leading to immunosuppression that increases fish susceptibility to pathogens affecting their survival and growth rates.

Coastal waters and lagoons are typical environments devoted to extensive aquaculture. In Italy, which annual production of extensive reared fish is nearly 5.250 tons, mullets represent the most important cultured species with an average production of about 3.000 tons per year. The coastal environments in which they grow are constantly exposed to high levels of urbanization and, consequently, to the action of increasing amounts of contaminants discharged in waters. This fact can play a central role in some emerging issues like mycobacteriosis and gonadal abnormalities in cultured mullets, representing a real concern for fish health and reproduction.

The term "mycobacteriosis" or "fish tuberculosis" decribes a chronic systemic and progressive disease caused by mycobacteria belonging to the genus *Mycobacterium*. In particular, *Mycobacterium marinum* is a slow-growing non-tuberculous mycobacterium and it is considered the most common etiologic agent of mycobacteriosis in wild and cultured fish. This disease is considered a real risk for fishermen and aquarists that manipulate infected fish. The diagnosis of mycobacteriosis is principally made by histology when positive Ziehl-Neelsen stain granulomas are detected. The aim of the first part of this study was to investigate the occurrence of mycobacteriosis in extensively cultured Mugilidae (*Chelon labrosus, Liza aurata, Liza ramada and Mugil cephalus*) of four lagoons from Sardinia by the use of histology, microbiology, PCR and DNA sequencing. Twenty-five out of 495 mullets (148 specimens from Cabras, 120 from Calich, 89 from Marceddì, and 138 from San Teodoro), collected during summer and autumn of the years 2013, 2014 and 2015, were suspected of being infected with mycobacteriosis revealing granulomas containing acid-fast bacilli at histopathological examination. In particular, 10 out of 25 mullets were certainly affected by mycobacteriosis and *Mycobacterium marinum* was identified in 6 out of 10 as the primary cause, and the concordance obtained by histology, cultural evaluation and sequence analysis of the hsp65 gene was 100%. In the remaining 4 specimens, *Mycobacterium* spp. were detected and the concordance obtained by histology and molecular method showed 100% of positivity. In the remaining 15 specimens,

granulomas with acid-fast bacilli were detected although culture confirmed the positivity for *Mycobacterium* spp. only in 6 cases, with an accordance of 43% with histology. In all of these cases, PCR-hsp65 and sequencing failed to identify atypical mycobacteria. Mullets affected by mycobacteriosis were mainly sampled in the Calich (10%) and San Teodoro (8%) lagoons. Only 2 subjects were positive for mycobacteriosis and no cases were observed in Cabras and Marceddì lagoons, respectively. This study confirms that histopathological examination is a very important diagnostic screening tool for the detection of mycobacteriosis in fish and PCR-hsp65 is a valid and easy method to identify atypical mycobacteria, expecially for *M. marinum*. Our finding are worthy of attention because mycobacteriosis in mullets has been evidenced for the first time in Sardinia, suggesting that this disease may be underestimated also in other cultured fish species. These results confirm our expectation that mullets living in San Teodoro and Calich lagoons, both characterized by critical environmental conditions, could play a central role in understanding the occurrence of fish mycobacteriosis which, if not properly monitored, could represent a serious concern for public health.

Fish are among the most studied organisms for the effects of chemical contaminants on the development and reproductive processes. In coastal and estuarine ecosystems, euryhaline fish living in polluted waters like Mugilidae can frequently show sexual anomalies like intersex. This term describes alterations in gonadal development with the simultaneous presence of male and female reproductive stages in the same gonad of a gonochoristic species. In the second part of this study, adult specimens of three species of euryhaline mullets (*Chelon labrosus*, *Liza aurata*, and *Mugil cephalus*), from two Sardinian lagoons (Marceddì and San Teodoro) devoted to extensive aquacultural practices, were analyzed in order to identify putative alterations in gonads and in gamete development. Overall, 13 of the 158 mullets examined (8.2%) were affected by gonadal disorders: four subjects (one *C. labrosus*, two *L. aurata* and one *M. cephalus*) exhibiting an intersex condition were found in the Marceddì lagoon and the other nine (five *C. labrosus*, two *L. aurata* and two *M. cephalus*) in the San Teodoro lagoon. Twelve of these gonads were classified as testis-ova (TOs) and one, belonging to a *C. labrosus* specimen, was a mixed gonadal tissue (MGT). Intersex condition was evaluated using an intersex index and all the recorded values showed a mild ovotestis severity index (OSI). However, our findings suggest that fish gonadal disorders may be underestimated in extensive reared fish species, particularly in coastal brackish

environments polluted by intensive agriculture and animal husbandry activities. In conclusion, further research on emerging fish disorders and diseases in Sardinian coastal lagoons could confirm the hypothesis that species like mullets have to be considered as biological sentinels to detect the degree of pollution in extensive aquaculture systems, with the purpose of reducing risks to animal and human health.

Chapter 1

General Introduction

1.1 Global fish production

Capture fisheries involve capture of wild organisms while aquaculture is based on the rearing of fish, crustaceans, molluscs and algae. The interplay between these two activities are numerous: many of the farmed species are also fished, and in many cases fishery and aquaculture, especially in lagoons and lakes are integrated and their boundaries are not always so well defined (Cataudella & Bronzi, 2001).

Global fish production has grown regularly in the last six decades and in 2012 fisheries and aquaculture activities supplied the world with about 158 million tons of product, of which 136 million were for human consumption (Fig. 1.1).

Fish consumption increased in the last 60 years from 9.9 kg to 19.2 kg per capita due to the world population growth, urbanization, and the improving of fish production technology (Table 1.1)

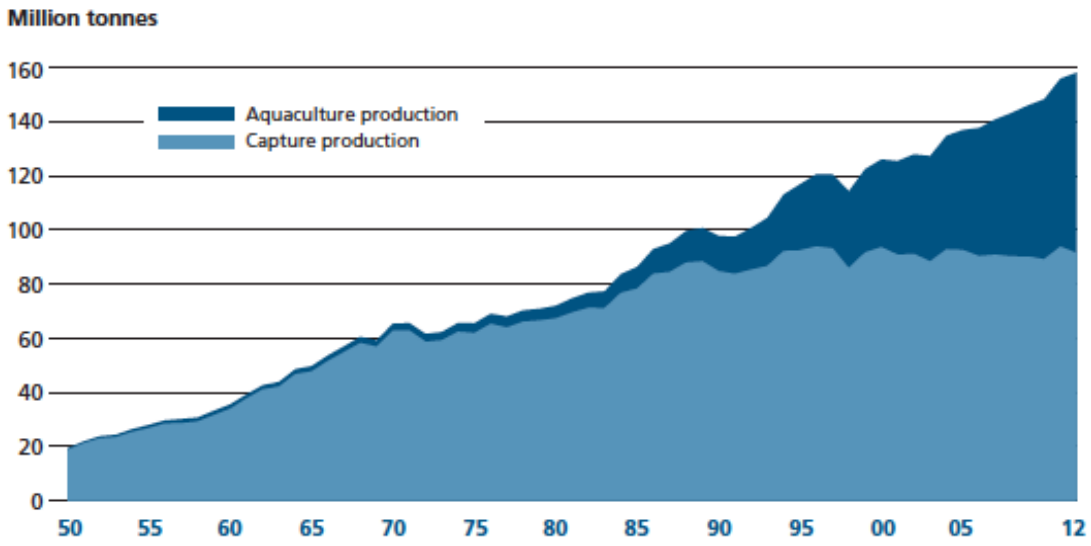

Fig. 1.1 Trend in world capture fisheries and aquaculture production from 1950 to 2012 (from FAO, 2014).

1.1.1 Production from capture fishery

Capture fishery production in 2012 was of 79.7 million tons in marine waters and of 11.6 million tons in inland waters respectively. Nevertheless recent data show that capture fisheries have reached the limits of growth and it is absolutely necessary that these activities do not exceed the limits imposed by the natural law (FAO, 2014).

Table 1.1 World fisheries and aquaculture production and utilization (from FAO, 2014).

	2007	2008	2009	2010	2011	2012
	(Million tonnes)					
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	**90.8**	**90.1**	**90.1**	**89.1**	**93.7**	**91.3**
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	**49.9**	**52.9**	**55.7**	**59.0**	**62.0**	**66.6**
TOTAL WORLD FISHERIES	**140.7**	**143.1**	**145.8**	**148.1**	**155.7**	**158.0**
UTILIZATION[1]						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (*billions*)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (*kg*)	17.6	17.9	18.1	18.5	18.7	19.2

Note: Excluding aquatic plants. Totals may not match due to rounding.
[1] Data in this section for 2012 are provisional estimates.

1.1.2 Production from aquaculture

FAO introduced a definition of aquaculture, which reduces its confusion with capture fisheries: "aquaculture is the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc." (FAO, 1988). In farming activities more than 600 aquatic species mainly fish, crustaceans and molluscs are cultured in different aquatic environments such as freshwater, brackish water and marine water. Aquaculture remains the productive activity showing the most rapid growth in the context of food production, with an average growth of 8.6% per year since 1980 (Costa-Pierce, 2002; FAO, 2014). Global aquaculture production of food fish has reached 66.6 million tons in 2012, where 44.2 were constituted of 38.6 million tons from inland aquaculture of finfish species and 5.6 million tons from mariculture (Table 1.2). In particular, growth of finfish is higher in inland than in mariculture and it is consequence of the easy-to-achieve type of aquaculture in developing countries.

Table 1.2 World production of farmed species groups from inland aquaculture and mariculture in 2012 (from FAO, 2014).

	Inland aquaculture	Mariculture	Quantity subtotal		Value subtotal	
	(Million tonnes)	*(Million tonnes)*	*(Million tonnes)*	*(Percentage by volume)*	*(US$ million)*	*(Percentage by value)*
Finfish	38.599	5.552	44.151	66.3	87 499	63.5
Crustaceans	2.530	3.917	6.447	9.7	30 864	22.4
Molluscs	0.287	14.884	15.171	22.8	15 857	11.5
Other species	0.530	0.335	0.865	1.3	3 512	2.5
Total	**41.946**	**24.687**	**66.633**	**100**	**137 732**	**100**

1.2 Aquaculture production systems and practices

Aquaculture developed varieties of system that range from very extensive to hyper-intensive techniques. Intensive aquaculture is based on the rearing of fish in inland tanks or in marine floating cages where human intervention is necessary for feed and stocks management (MIPAAF, 2014). Extensive aquaculture cover large areas of water and characteristic environments are lagoons, delta river, estuaries, bays and ponds of inland areas generally near to the coasts. (Anras *et al*., 2010). This farming technique is practiced using freshwater or brackish water basins that cover extended areas. Generally cultured fish spend their earlier life in marine or freshwater and only the last part of rearing occur in brackish waters where the level of salinity varies from 0.5% to seawater (FAO, 2016). Extensive aquaculture is the closest farming methods to fishing and is regulated by minimal human intervention limited to simply catching aquatic organisms with artisanal fisheries as fixed capturing systems, nets or hand weapons (Cataudella & Bronzi, 2001).

More specialized methods are planned in different parts of productive cycle such as managing interventions, diet integration and control of juveniles (Bostock *et al*., 2010). However traditional practices may present differences moving from one country to another changing farming protocols and water management (Anras *et al*., 2010). Extensive aquaculture, that exploits mostly lagoon resources, constitutes a traditional model where general structures like hydraulic barriers as weirs and locks are used (Cataudella & Bronzi, 2001). Generally the management of the lagoon is organized in small farms where fishing license is owned by one or few cooperative that handle and sell fish stocks.

1.2.1 Extensive aquaculture in the Mediterranean

Several forms of aquaculture including extensive systems are now an important reality of fish production in the Mediterranean coastal lagoons and are part, from long time, of the cultural tradition of the regions, with around 400 coastal lagoons, covering a surface of over 641.000 ha. Productivity varies from a few to several hundred kilograms per hectare per year (kg/ha/year) based on the lagoon typology and ecology, although for many different reasons achieve consistent and updated fish production is still a challenge (FAO, 2015a).

Territory characteristics and several reared species (mainly seabream, seabass, mullet, sole and eel) are distinctive of different European regions. In a recent report, European Commission declares that aquaculture can still grow providing new job opportunities and gradually satisfy consumer internal request of safe and sustainable seafood products. Moreover in that report is observed that the increase of human activities (urbanization, tourism-related facilities, wastewaters from industrial and agricultural activities) have a harmful effect on this ecosystem and is becoming one of the major restraint to progress of the extensive aquaculture in Europe (European Commission, COM (2013) 229 final).

1.2.2 Extensive aquaculture in Italy

In Italy 198 lagoons, coastal lakes and ponds are present for a total surface of 167.575 ha, of which 40.000 are dedicated to extensive aquaculture. Lagoons are distributed mainly in four Italian geographic zones largely diverse in morphology and ecosystem: the northern Adriatic, the south Adriatic, the central Tyrrhenian and Sardinia and Sicily. (Fig. 1.2).

The extension of brackish lagoons that range from few ha to 1.660 ha, in the North of Italy (Veneto, Emilia-Romagna and Friuli-Venezia Giulia) are named "valli di pesca" and represents confinement of portion of coastal lagoon (vallicoltura) (Cataudella & Bronzi, 2001; MIPAAF, 2014).

Extensive aquaculture represents the 12,4% of national seafood production, with a production amounts ranging from 40 kg/ha of Lesina lagoon to 319 kg/ha of Sardinian lagoons (Cataudella & Bronzi, 2001).

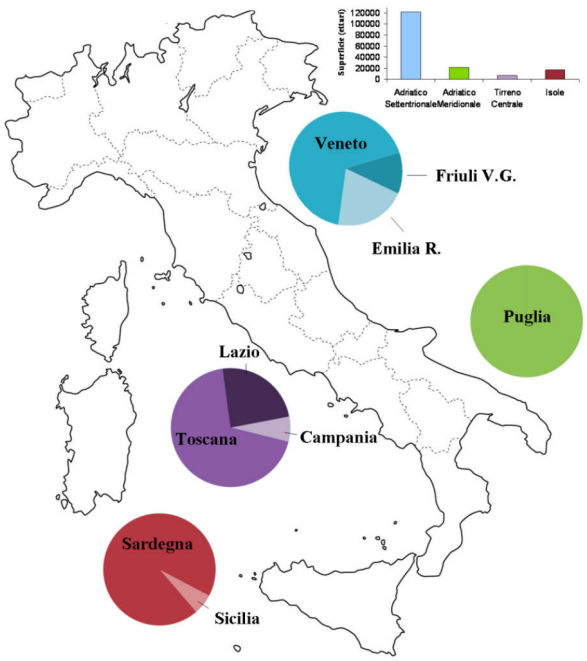

Fig. 1.2 Lagoons in Italy: surface in the four areas mainly devote to extensive aquaculture (histogram) and relative distribution in the nine regions (circles) (FAO, 2015a).

Italian production from lagoon environments is highly variable, and data began to be collected from 2008. FAO reports 2.787 tons of fish and 35.007 tons of shellfish for 2009 (FAO, 2015a and reference therein). Although lagoon system possesses a valuable biodiversity and richness in aquatic animals, only few species are of commercial interest. These generally include Mugilidae species, such as *Chelon labrosus* (thicklip mullet), *Liza aurata* (golden grey mullet), *Liza ramada* (thinlip mullet), *Liza saliens* (leaping mullet), and *Mugil cephalus* (grey mullet).

Other common fish species are *Sparus aurata* (seabream), *Dicentrarchus labrax* (European seabass), *Anguilla anguilla* (European eel), *Atherina boyeri* (Boyer's sand smelt) (Cataudella & Bronzi, 2001).

With a total national production of 5.250 tons of extensive reared fish, mullets represent the most important productive sector as they account for the 57% of total, with an annual production of 3.000 tons. Moreover seabream and seabass are largely diffuse and contribute for the 16% (850 tons) and 13% (700 tons) of national production, respectively; eels complete lagoons production with a percentage of 13% (700 tons) (MIPAAF, 2014) (Fig. 1.3).

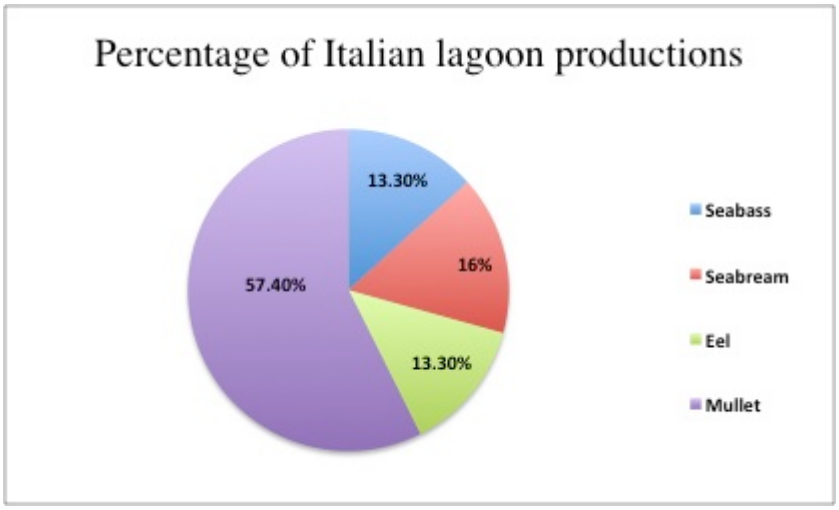

Fig. 1.3 Extensive aquaculture productions in Italy: mainly fish species farmed and their relative percentage (MIPAAF, 2014).

1.2.3 Extensive aquaculture in Sardinia

Sardinia has one of the most extended area of lagoons and ponds of Europe. According to Fenza *et al.* (2014), lagoons identify a coastal basin, characterized by brackish water, separated from the sea only by thin land boundaries that permit good water circulation, whereas in ponds exchanges with the sea are absent or modest and the water circulation is slower (Fenza *et al*., 2014).

Of the 77 coastal lagoons, covering a total area of around 15.000 ha, only 27 are now used for extensive aquaculture. These cover a total area of 5.700 ha, with 3.800 ha concentrated on the central western coast (Oristanese), where the most important lagoons are in Cabras (OR, 2.230 ha) and in S. Giovanni-Marceddi (OR, 1.600 ha). (Cataudella & Spagnolo, 2011; Fenza *et al*., 2014).

The other lagoons are present in the North-western area of Sardinia (Nurra), North East (Gallura -Baronia), South East (Ogliastra-Sarrabus-Gerrei), South (Cagliaritano) and South West (Sulcis-Iglesiente) as reported in Fig. 1.4.

Extensive aquaculture in Sardinia is practiced in lagoons and ponds with simpler technology compared to the extensive poly-culture method like “vallicoltura”. Generally in Sardinia production practices are based on traditional methods by using fixed capture systems generally V-shaped chambers called “lavorieri” built in wood, reeds, concrete or plastic (Fig. 1.5).

Fig. 1.4 Sardinian wetland areas used for extensive aquaculture (from Fenza *et al*., 2014).

Fig.1.5. Typical plastic V-shaped "lavoriero".

"Lavorieri" lead the fish moving from the sea towards the lagoon where they find abundance of food and a more protected environment than marine waters. These

systems permit to capture fish when they migrate from the lagoon back to the sea during their reproductive cycle (FAO, 2015a). In Sardinian wetlands are also diffuse different types of fishing with traps and nests to catch principally eels, gobies, crabs and cuttlefish (Fenza *et al*., 2014).

Among the many aquatic organisms that can find a proper habitat in wetlands, very few fish species of commercial interest are mainly extensively cultured in Sardinia: mullets and eels are the most represented, but also crabs and sand smelts. In waters where salinity is higher are captured seabreams and seabasses, the most valued species, but also gobies, red mullets and flounders can be found in these lagoons.

Sardinian ponds appear to be the most productive lagoons in Italy reaching a mean production of 50 kg/ha (a minimum of 25 and a maximum 320 kg/ha/year) (Cataudella & Bronzi, 2001). However Sardinian lagoons production have reached in the 1980s 600 kg/ha in the lagoon of Tortolì.

Many causes may play a role in determining these large differences in productivity. One can be attributed to the progressive changes of salinity and water temperature that may alter the ecological equilibria of wetlands as has been recorded in some ponds in Sardinia. Anyway pollution is becoming a serious problem having caused severe diseases in fish of many lagoons like in Cabras, Santa Giusta (OR) and San Teodoro (OT) (Cataudella & Spagnolo, 2011).

It is interesting to note how the property of wetlands is allocated in Sardinia. The "Regione Autonoma della Sardegna" (R.A.S.) is owner of twenty-four production ponds that give in concession to fishing cooperatives that can manage and sell fish stocks coming from those lagoons.

Only three ponds are privately owned (Fenza *et al*., 2014). However fish productions could be higher if implants could receive a relevant economic effort of modernization. This element is of great importance when you consider that the combination of fishing and aquaculture and their strong integration represent the future of this activity to safeguard the quality of products and for the safeguarding of resources. The combination of responsible fishing and sustainable aquaculture is considered to be the framework for the proper management of environmental resources.

1.3 Generalities of mullets

1.3.1 Mullet species in the Mediterrenean Sea

Fish of the family Mugilidae, class Actinopterygii, order Mugiliformes, includes 20 genera and 71 species (Eschmeyer & Fong, 2016; Eschmeyer et al., 2016). Four genera (*Chelon, Liza, Mugil* and *Oedalechilus*) and six species of mullets (thicklip mullet *C. labrosus*, golden mullet *L. aurata*, thinlip mullet *L. ramada*, sharpnose mullet *L. saliens*, flathead mullet *M. cephalus*, boxlip mullet *O. labeo)* are endemic in the Mediterranean Sea (Whitehead, 1984; FAO, 1995; Nelson, 2006; Turan, 2007).

1.3.2 Morphology and morphometry

Although general features such as fusiform body, large scales, grey coloration on upper back and silver grey along flanks are common traits in Mugilidae, only some morphological characters are suitable to classify among different mullets species as reported by Farrugio (1977) and by Turan *et al*. (2011) see Figs. 1.5, 1.6 and 1.7, and Table 1.3 respectively.

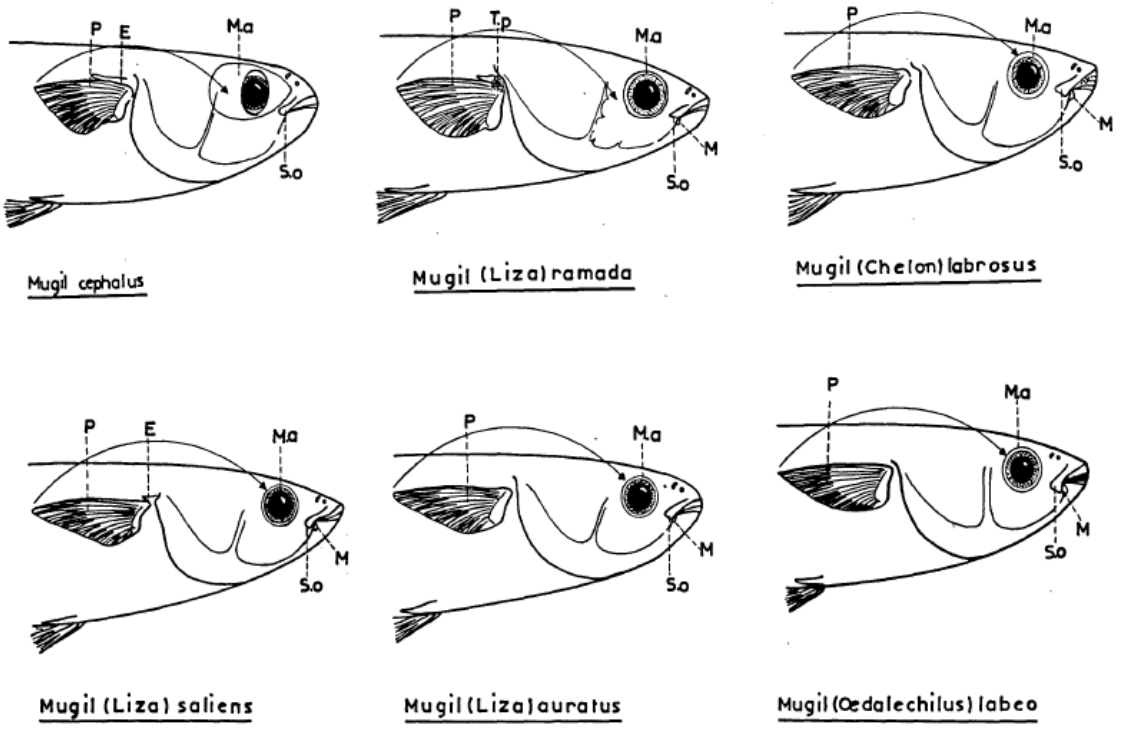

Fig. 1.6 Profiles of mullet heads. E: axillary scale. M: maxillary. Ma: adipose membrane. P: pectoral fin. So: sub orbital. Tp: pigment spot (from Farrugio, 1977).

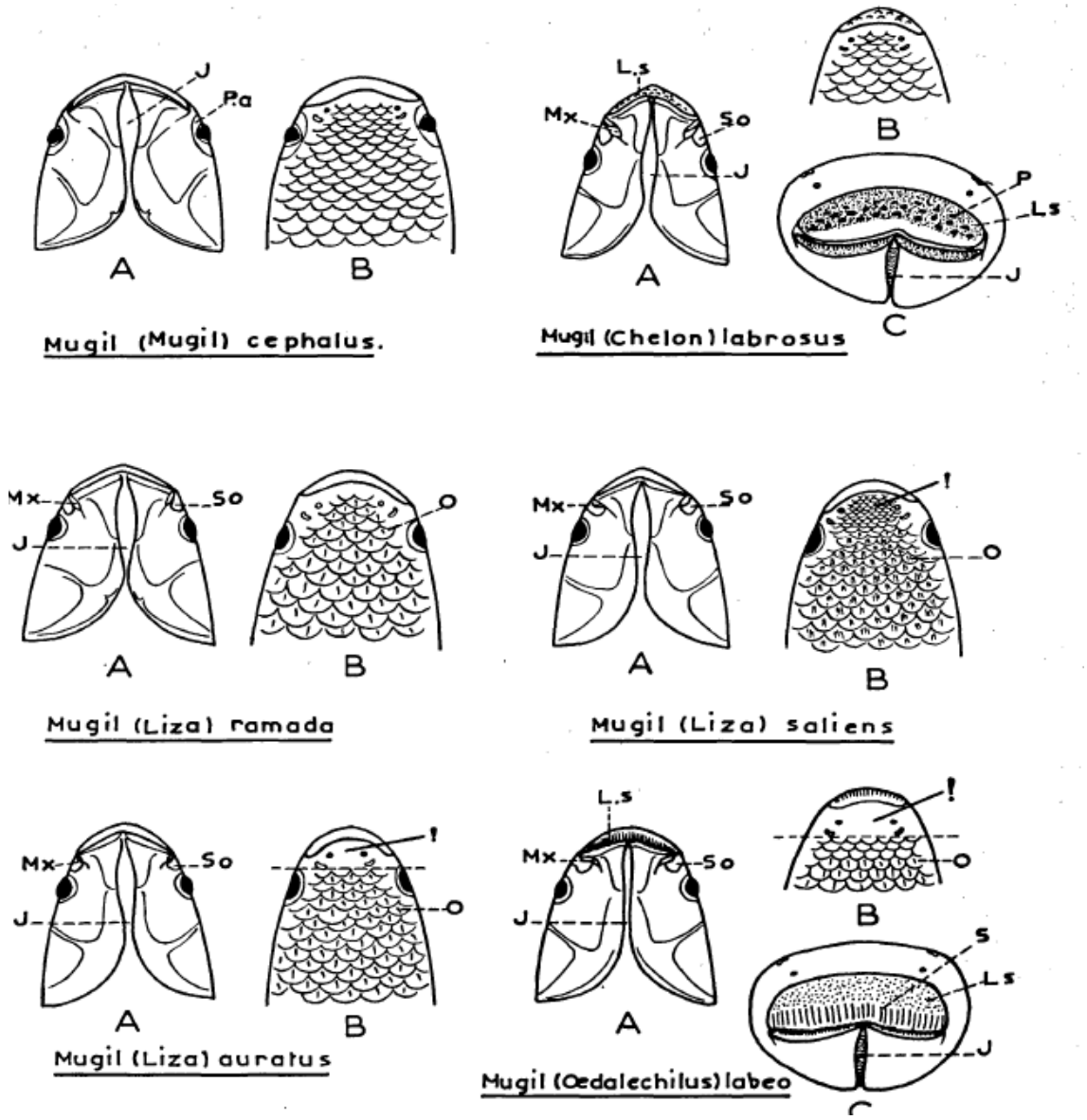

Fig. 1.7 Mullet heads. A: ventral face. B: dorsal face. C: frontal face. J: jugular space. LS: upper lip. Mx: maxilla. O: scales. P: papillae. Pa: adipose eyelid. So: sub orbital (from Farrugio, 1977).

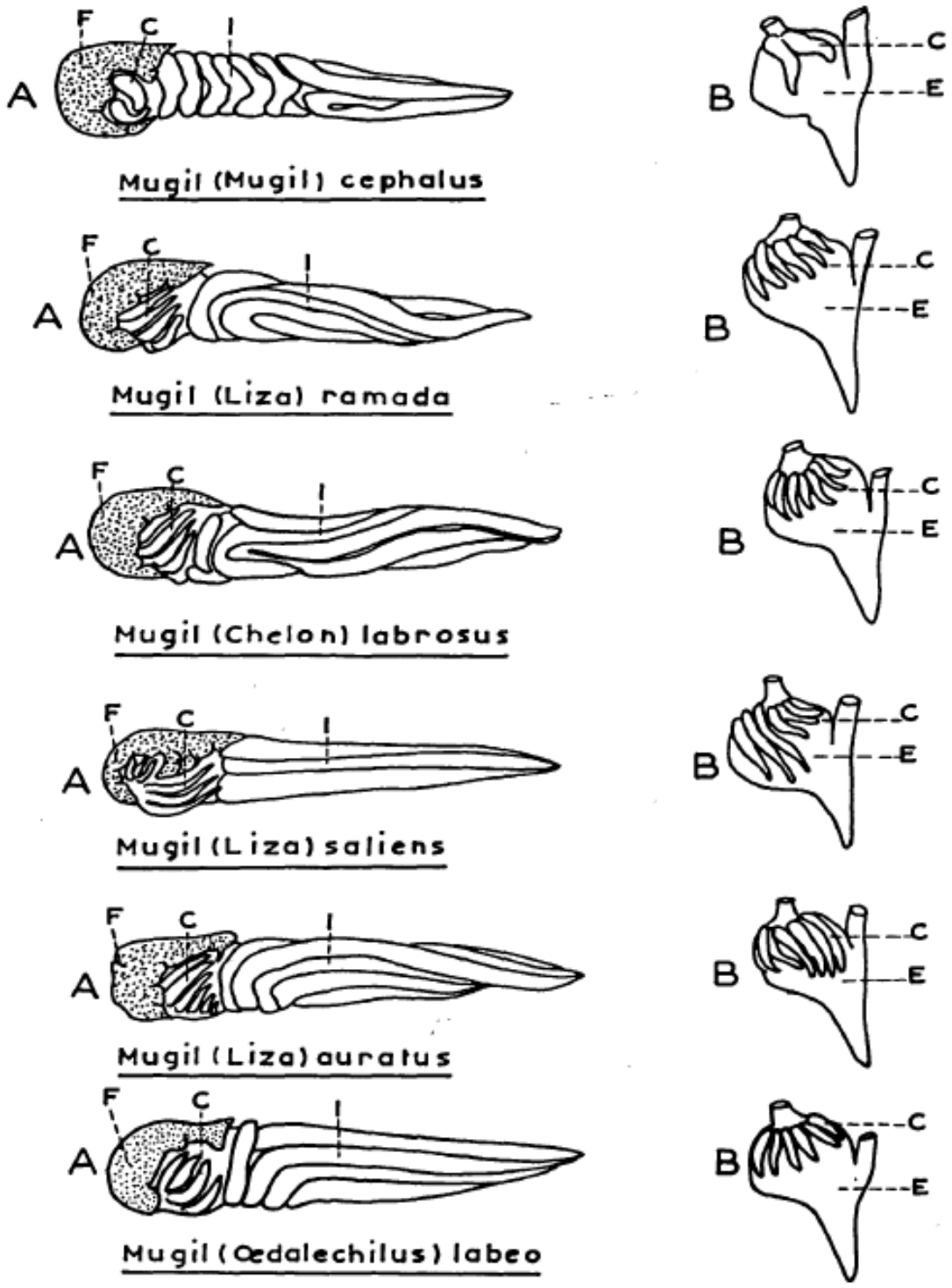

Fig. 1.7 Scheme of intestinal tract of mullets and profile of stomach. C: pyloric caeca. E: stomach F: liver. I: intestine (from Farrugio, 1977).

Table 1.3 Descriptive taxonomic characters used to distinguish Mediterranean mullets. First dorsal fin rays (DFR1), second dorsal fin rays (DFR2), ventral fin rays (VFR), anal fin rays (AFR), pectoral fin rays (PFR), pyloric caeca (PC) (from Turan *et al.*, 2011).

Species	DFR 1	DFR 2	VFR	AFR	PFR	PC
Chelon labrosus	IV	I 8	I 5	III 8–9	17	6–7
Oedalechilus labeo	IV	I 8	I 5	III 8–10	16–17	6
Mugil cephalus	IV	I 8	I 5	III 8–9	17	2
Liza haematocheila	IV	I 8–9	I 5	III 8–9	16	4–5
Liza aurata	IV	I 8	I 5	III 8–9	16	7–8
Liza carinata	IV	I 7	I 5	III 7	15	5
Liza ramada	IV	I 7–8	I 5	III 8–9	16–17	6–8
Liza saliens	IV	I 7	I 5	III 7–8	16	7–9

However, in Mugilidae species the morphoanatomy is so similar and at the same time the evolutionary tool is not so straight in interpreting anatomical differences. Hence classifying species and genera in terms of phylogenetic inferences is problematic. Fortunately the extensive developments of Polymerase Chain Reaction (PCR) offer a valid support into the study of the phylogeny and diversity of the Mugilidae families and species (Durand *et al*., 2012).

1.3.3 Food and feeding

Mugilidae, generally known as mullets are distributed in all tropical and temperate seas, living in offshore and coastal waters, lagoons and estuaries (Boglione *et al.,* 2006; Fenza *et al.,* 2014). Mullets are euryhaline species able to adapt to a wide range of water salinities and to live both in fresh water (FW) than in sea water (SW) at different salinity levels. In particular some mullet species well tolerate FW and others inhabit waters more saline than normal SW. Euryhaline fishes need to maintain a particular homeostasis of their internal fluids and gills, alimentary tract, kidneys and neuroendocrine. This latter is the major organ system involved in osmoregulatory functions in mullets (McCormick, 2001).

Mullets are able to get feeding from water surface to mud bottom and have been named as mud or detritus feeders (Brusle, 1981). Diet consists mainly of organic matter suspended or present in sediment, benthic invertebrates, macroalgae and plankton. Benthic animals are generally present in the sediment and constitute the major source food of mullets. They are classified into meiofauna, macrofauna and epifauna (Cardona, 2016). For that reason mullets live in ecosystems where the sediment is plenty of

organic matter, like coastal lagoons and estuaries (Cardona *et al.*, 2001).

The gastroenteric apparatus of young and adults mullets became similar when juvenile move from a zooplanktophagous to sedimentivorous diet (Ebeling, 1957; Albertini-Berhaut, 1987).

Mullets have a specialized feeding mode different than other species of fish. They orient their head down when eating by using their powerful jaw to scrape the bottom surface assuming sediment and associated food material (Odum, 1970; King, 1988). In particular they have toothed mouth to scrape microbial films, teeth and gills to remove large and small particles respectively and a stomach with a powerful gizzard (Ebeling, 1957; Capanna *et al.*, 1974). Different oral and pharyngeal anatomical structures are also reported in mullets species: i.e. *Chelon labrosus* usually feeds organic debris and algae by using tubercles of its upper lip, *Liza* genus prefers small particles in soft mud while *Mugil cephalus* eat larger particles in sandy substrates (Boglione *et al.,* 2006). However, the food quantity and quality vary considerably seasonally and during spawning migration some mullets species stop feeding (Odum, 1970; Cardona, 2001).

1.3.4 Age and growth

Growth in fish, the increase in length or weight directly related to age is a complex mechanism in which several factors and variables play a role. As is known, growth is considered as a process mostly driven by fish physiology strictly related to season, habitat, food, sex, water temperature, salinity, fish health etc. (Froese, 2006). Regarding mullets, authors report that a very rapid growth rate during the first two years of life is observed while reducing significantly in the rest of life (Brusle, 1981; Abbas, 2001). The greatest growth rate is reported in *Mugil cephalus* respect to the genus *Liza* and *Chelon labrosus*. Low temperatures and scarce availability of food are considered causes that may influences mullet growth. In addition reproductive period is known as a critical event in mullet life because storing of reserves for spawning migrations (Alvarez-Lajonchere, 1982). Mullet body structures, which proportionally increase with lifetime, i.e. natural rings on scales and otoliths are useful to calculate age (Morales-Nin, 1987). The first ring appears between one year and one and a half years of age (Hotos, 2003; Abdallah *et al.,* 2012) and also environment factors such as food availability and temperature have been observed to influence the subsequent deposition rate of rings (Panfili *et al*., 2002). However the question to determine mullet age is still an open problem (Hsu & Tzeng, 2009).

1.4 Reproductive state of mullets

1.4.1 Sexuality

Reproduction in fish is regulated by neuroendocrine system. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and liberation of gonadotropins from the adenohypophysis. Some of them are related to the reproduction i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The function of LH and FSH is still unknown even it is proved their action on the gonads to induce sex steroid productions involved in maturation of gametes (Devlin & Nagahama, 2002). FSH in female stimulates the production of 17β-estradiol by the ovary and induces vitellogenesis, while in males stimulates Sertoli cells proliferation and spermatogenesis. The value of LH is high in oocyte maturation and spermiation, whereas is undetectable at the first stages of gonadal development. The hypothalamus- pituitary-gonadal axis and the liver system regulating reproductive function in mullets are also influenced by environmental factors like temperature, photoperiod and water salinity (Cerdá-Reverter & Canosa, 2009). Mullets are species with sexes separate (gonochoristic) and with no sexual dimorphism so that distinguish between sexes are not feasible. Gonads are first undifferentiated and later develop into ovary or testis. Reproduction consists of gametes released into the coastal water and with an external fertilization (Kjesbu, 2009). The gonad shows two different types of cell named germ and somatic cells. In the ovary somatic cells develop into granulose and thecal cells, whereas in the testis differentiate into Sertoli and Leydig cells. Thecal and Leydig cells produce sex steroid hormone (Pandian, 2012). Testis grows in size and vascularization along with the increasing mullets length. The testis of mullets contains seminiferous tubules lined with a layer of germ cells that develop into spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa with four stages of sperm cell maturity (immature, maturing, functional maturation, and recovering stage) (Albieri & Araújo, 2010). The sperm move into the seminiferous tubules to the epididymis and then through the vas deferens to the urethral orifice (Guimaraes-Cruz *et al.*, 2005). Folliculogenesis begin when oogonia differentiating into primary oocytes. In mullets, primary oocytes are small (80 to 100 μm) and relatively uniform in size (McDonough *et al.*, 2005). At the beginning of reproductive development the oocytes increase in size and arrest at the first meiotic division (Stenger, 1959). Ovaries, when differentiated, increase vascularization and ovarian-cross section become more rounded in shape.

Regarding reproduction activity mullets are reported as isochronal spawning fishes that

spawn gamete, synchronously developed, at the same time or over a very short interval period (Greeley *et al.*, 1987; Render *et al.*, 1995). Nature of spawning in sea mullet is still controversial and although differences between various Mugilidae species are present, they spawn once a year in autumn-winter, winter-spring or summer (Fenza *et al.*, 2014). Maturity stage of ovaries is a way to determine the duration of the reproductive season of mullets that is defined as the time between the beginning and the end of spawning event.

This latter has been evaluated by using gonadosomatic indices (GSI) that indicate reproductive stage and the timing of spawning. However GSI is still questioned because reproductive activity may be not reliable in case of young female or individuals with anomalous ovarian development (Smith & Deguara, 2002).

Therefore additional evaluation of gonad maturity should include the classification of oocytes stage by histology that could permit a greater accuracy in determines the gonad maturity. Moreover the availability of histological data can be used to relate the GSI values with oocyte growth stages.

1.4.2 Oocyte development

Different classifications have been assessed to stage oocyte growth because immature ovarian germ cells (oogonia) develop into mature oocytes trough changes in their nuclear and cytoplasm structure based on two principally maturations referred as previtellogenic and vitellogenic stages (McMillan, 2007 and references therein).

- *Primary growth (previtellogenic stage)*

  Nucleolar morphology characterizes two phases of primary growth of oocytes in fish: the chromatin-nucleolus phase and peri-nucleolus phase. In the first phase oocytes have scarce cytoplasm and a large nucleus, centrally located, which usually contains a single large basophilic nucleolus. Oocytes in this phase arrest their growth in diplotene of the first meiotic division. In the peri-nucleolus stage oocytes show basophilic cytoplasm and nucleus increases in size to form the germinal vesicle while nucleoli move to the periphery of the nucleus (McMillan, 2007 and references therein).

- *Secondary growth (cortical alveolus stage)*

  The cortical alveolus stage is characterized by the presence of a clearly visible “zona radiata” while in the cytoplasm are observed empty spherical structures (lipid

vacuoles) called alveoli that increase in number and growth on the periphery of the oocytes. Proteins such as vitellogenin (VTG) synthesized endogenously are accumulated into the cortical alveoli and during fertilization the content is released within the vitelline membrane to preclude polyspermy and the ingress of pathogens.

- *Vitellogenesis*

This stage is characterized by the deposition of VTG, the main precursor of yolk proteins, into oocytes (Tyler & Sumpter, 1996). VTG is an extraovarian plasmatic phosphoglycolipoprotein produced by the fish liver that plays a central role during the early embryo development (Babin *et al*., 1999). In vitellogenesis stage follicular cells increase in number and granulosa and theca cells as well as "zona radiata" are more evident.

- *Maturation (germinal vesicle migration; hyalinization; hydration)*

This phase is characterized by final oocyte maturation (hydration) and progression of meiosis. Although scarce reports are documented about final oocyte maturation and ovulation in mullet species, the ovulatory process includes numerous modifications in the oocytes and ovarian follicle (Lemos *et al.*, 2014). Follicular rupture caused by proteolytic enzymes digestion and hydration processes determine the expulsion of the oocyte and is regulated partially by prostaglandins (Khan & Thomas, 1999).

1.4.3 Postovulatory follicles

After the oocyte is expelled the postovulatory follicles (POFs) are reabsorbed in 72-96 hours. Ovary at this stage can indicate a recent spawning as reported in few species of Mugilidae (Hsu *et al*., 2007; Lemos *et al*., 2014).

1.4.4 Atresia

The oocyte degeneration is called atresia and any stage of oocytes development can be affected. Atretic oocyte does not have the nucleus, show yolk granules and disruption of the vitelline membrane. Factors like environmental stress may increase the occurrence of follicular atresia negatively affecting fecundity (Sharma & Bhat, 2014).

1.4.5 Ovarian maturity

In mature ovaries, mullets have two groups of oocytes at the same time: synchronous

oocytes and small oocytes (previtellogenic), which are indicative of a conservative reproductive state of this species (González-Castro *et al.*, 2011). To determine accurately the potential of fecundity, female mullets must be recognized as immature or mature, taking into account only maturity ovaries with no sign of recent spawning or atresia. Fecundity of mullets depends on various parameters like species, geographical area and season and generally increases with growth in size and age of the females (Brusle, 1981; Alvarez-Lajonchere, 1982; González-Castro *et al.*, 2011). Ovarian stages maturity has been macro and microscopical studied and authors identified four to seven stages of ovarian maturity in Mugilidae (El-Halfawy *et al.*, 2007; González- Castro *et al.*, 2011; Lemos *et al.*, 2014). Ovarian stages are indicated as follow:

1. Virginal: translucent ovaries, weight less than 1 g with oogonia and few primary growth oocytes.
2. Immature: pink ovaries, weight between 1 and 3 g with primary growth oocytes.
3. Incipient maturity: pale to dark yellow ovaries; weight 10 to 20 g with primary growth and cortical alveoli oocytes. Females in this stage are considered as mature.
4. Advanced maturity: dark yellow to orange ovaries with prominent ovarian artery, weight between 30 and 280 g with rare primary growth oocytes and numerous yolked oocytes.
5. Spawning: translucent ovaries occupying the entire abdominal cavity and with oocytes ovulated.
6. Spent: reddish to flaccid ovaries occupying the 25% of the abdominal cavity with residual ovulated oocytes.
7. Resting: reddish or greyish ovaries, weight 4 to 10 g with primary growth oocytes and oogonia.

1.5 Morphometric indices

Several morphometric indices have been identified as potentially good indicators of the general well-being of wild and cultured fish. Among these, the following are the most used.

1.5.1 Fulton's condition factor

The Fulton's condition factor (K) is determined by the formula

$$K = (W/L^3)\times 100$$

where W is somatic weight (grams) and L is fish length (centimetres). Somatic weight is calculated as the total weight of fish less gonad and stomach content weights. Somatic weight is selected, as feeding intensity and gonad maturation can vary significantly and independently of condition between seasons and within and between stocks (Lambert & Dutil, 1997). Somatic weight is also a more precise reflection of condition, as available energy reserves of an individual will be located in somatic tissues (somatic cells as opposed to germ cells).

1.5.2 Epatosomatic index

Another important morphometric index is the hepatosomatic index (HSI), which is calculated by the formula

$$HSI = (LW/TW)\times 100$$

where LW and TW represent liver weight and total weight (grams), respectively.

1.5.3 Gonadosomatic Index

The gonadosomatic index (GSI) is an efficient tool for determining the reproductive condition of mullets. The physiological state of the gonads can be estimated through the ratio between the gonad weight (GW) in grams and the total body weight in grams (TW).

$$GSI = (GW/TW)\times 100$$

GSI has different average values between male and female and GSI has the highest values in the spawning season when mullet migrate to the sea. This is related in female to the remarkable growth in volume and weight of oocytes during the spawning season, whereas the male gonads do not have significant variation (Okumus & Bascinar, 1997; Hotos *et al.*, 2000; Albieri & Araújo, 2010; Lemos *et al.*, 2014). Highest mean-gonadosomatic index (GSI) obtained for species of Mugilidae in different regions are reported in Table 1.4.

Table 1.4 Highest mean-gonadosomatic index (GSI) obtained for the species of Mugilidae (from Crosetti & Blaber, 2016).

Species	Region	Highest mean-GSI (%)		Month	Reference
		Females	Males		
Liza aurata	Messolonghi, W. Greece	5.32	2.78	September	Hotos et al. (2000)
-	Caspian Sea, Iran	6.4	2.7	October	Ghaninejad et al. (2010)
Liza ramada	Göksü Delta	16	-	November	Ergene (2000)
-	Lake Timsah, Egypt	12.4	-	November	El-Halfawy et al. (2007)
-	Adriatic Sea coast, Croatia	8	-	October	Bartulovic et al. (2011)
Liza saliens	Southeast Caspian Sea	5.9	2	June	Patimar (2008)
Liza klunzingeri	Arabian Gulf, Kuwait	7.5	5	November	Abou-Seedo and Dadzie (2004)
Valamugil cunnesius	Negombo lagoon, Sri Lanka	11.88	1.09	March	Wijeyaratne and Costa (1988)
Mugil cephalus	Taiwan	18	12	January	Su and Kawasaki (1995)
-	Jeju Island, Korea	6.97	-	December	Kim et al. (2004)
-	South Carolina estuaries, EEUU	17	11	November	McDonough et al. (2005)
Rhinomugil corsula	Meghna River, Bangladesh	5.5	-	July	Akter et al. (2012)

1.6 Current state of mullet culture

Mullets are species that at juvenile stage migrate from water sea to the costal lagoons and estuaries in order to grow and feed. Based on this characteristic the extensive aquaculture exploits the moving of young mullets towards coasts and the returning of adults to the sea during the breeding season leading to their capture. Mullet species have a different migration season and fry schooling together are easily caught at different times of the year. In specialized extensive culture they are collected and used for restocking purpose following specific rules (Koutrakis, 2015).

In some parts of Italy, these activities called “novellame per allevamento” are permitted in spring (from March to June) and in autumn (from September to December). The Ministry of Agriculture and Forestry Policies allows mullet fry capture in Italy through licence. Several licences were issued in 2014 in Italy, though in Sicily and in Sardinia mullet fry capture is no longer authorized. Fry capture can be considered as depletion in marine resources by a decrease of recruitment to wild stocks, at the same time wild fry and juveniles farmed in costal lagoons have a greater chance to survive with respect to wild (Ciccotti & Franzoi, 2001).

However these two remarks recommend an approach governed by sustainability criteria (IUCN, 2007). Mullet culture had great expectation during the 70s and 80s with a flourishing of interest and investments but the difficulties encountered to control their entire life cycle in restricted environments reduced the attention that was eventually pointed to other species. The above remarks and the low price of flesh mullets is still a

critical point leading many countries to abandon mullets culture to make more profit with different fish species in aquaculture activities. However culture mullets, because of their feeding at a low trophic level, could provide an alternative chance considering the progressive reduction of marine resources.

1.6.1 Global production of mullets

The ecological relevance of Mugilidae species cannot be underestimated and they constitute for human populations an important source of food in certain parts of the world (Whitfield *et al.*, 2012). In some regions of the Mediterranean, mullets represent a diffuse commercial fishes both captured and extensive cultured (Turan, 2016). World mullet production has progressively increased in the last decade 2004-2013 (Fig. 1.8). Recent data from FAO reported that the production of Mugilidae from capture fisheries was 560.150 tons in 2013, whereas from aquaculture was 138.143 tons corresponding to 19.2% of world mullet production.

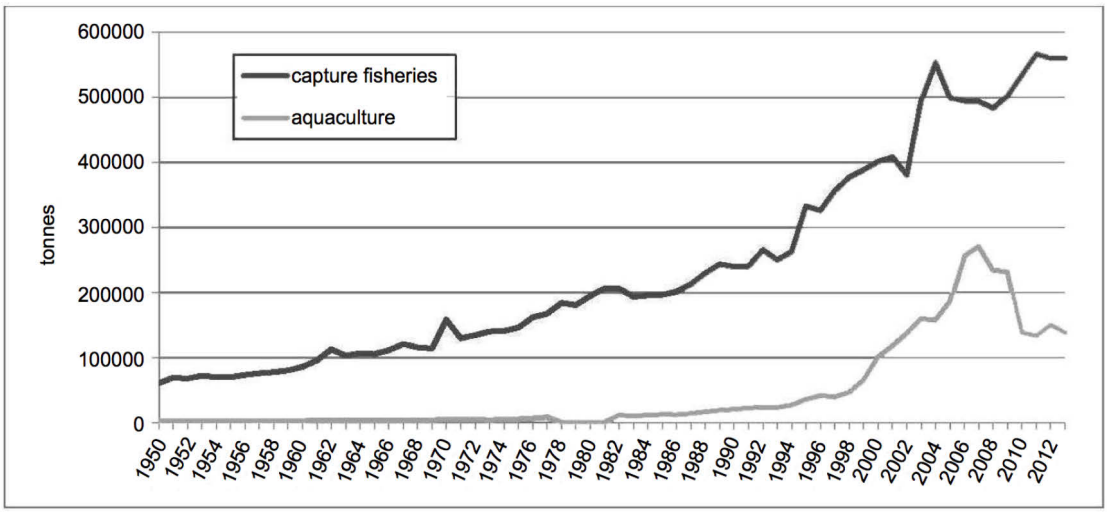

Fig. 1.8. World grey mullet production from captures and aquaculture (1950-2013) (from FAO, 2015b).

Mullet are cultured both in inland (both freshwater and brackish water) and marine areas (both brackish water and marine) and Africa is the highest producer of mullet among continents reaching the 84% of the world aquaculture production as showed in Fig. 1.9. In 2013 in Italy, mullet production from aquaculture was 530 tons of which 380 tons were of *Mugil cephalus* and 180 tons was coming from 'mullet nei' (Mugilidae) (FAO, 2015b). *Mugil cephalus* and *Chelon labrosus* are the species with

the highest price and the larger commercial size (Cataudella & Monaco, 1983). However production data regard the different species of mullet are not available in official statistics (FAO, 2015b).

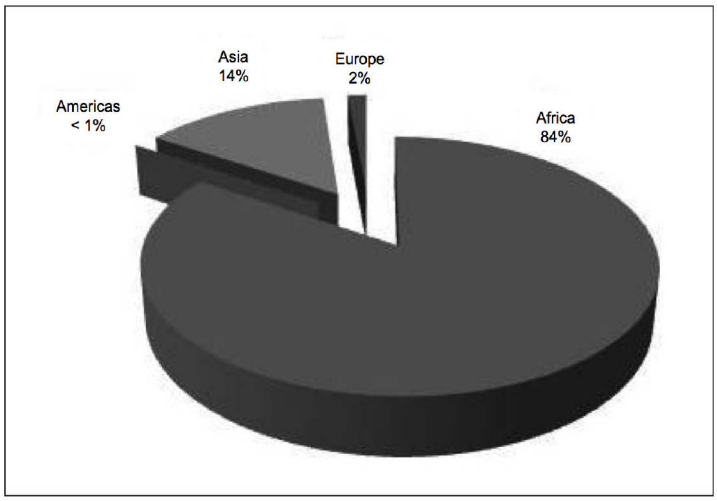

Fig.1.9 Mullet production from aquaculture in 2013 by continent (FAO, 2015b).

1.6.2 Products from mullets

The commercial interest of mullet is due to their flesh, which have different prices related to species, but in particular to egg roe considered traditional delicacies and sold at very high prize (Liao, 1981). Among mullet *M. cephalus* is the most commercially important species especially for egg roe that in Sardinian and in other Mediterranean regions are traditionally salted and dried (named bottarga) and represents an important income in the economy of aquaculture activities (Katselis *et al.*, 2005). Bottarga can be sold as whole roe sac vacuum or grated dry and constitute a source rich in poly-unsaturated fatty acids (n-3 PUFA) (Rosa *et al.,* 2009).

Factors such as size, shape, colour, salinity influence the quality and the price of bottarga, although mullet ovaries of different species of unknown origin and much cheaper than *Mugil cephalus* were mixed together as reported in Sardinia by Murgia (Murgia *et al.*, 2002). However mullet roe are still a not recorded product sold trough local and traditional trades.

1.7. Interaction between environment pathogens and fish health

Fish are subject to pathogens and non-infectious agents (i.e. genetic anomalies, metabolic disorders and stress environments) that affect fish survival and growth rates

causing fish disease (Sparks, 1972). Fish are constantly exposed to infection in water containing potentially pathogenic agents but disease is generally linked to an imbalance of pathogen virulence, resistance of the fish, and environmental stresses (Snieszko, 1974). Disease state is strictly related to changes of these factors as reported in Fig. 1.10.

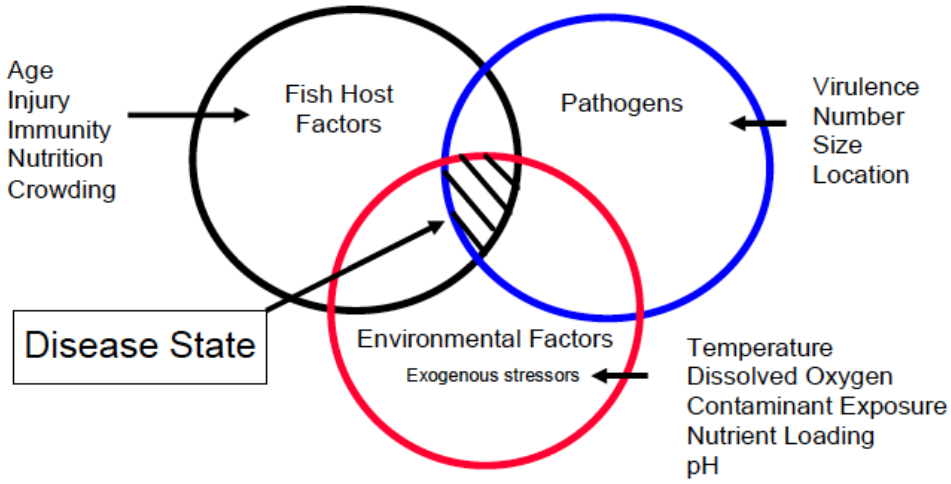

Fig. 1.10 Multifactorial nature of fish health issues. The interaction between environment, pathogen and host causing fish disease (www.cbf.org/Document.Doc?id=153).

Equilibrium between fish, pathogens and environments is generally observed in unpolluted waters where the possibility to develop diseases is certainly lower than in contaminated aquatic environment where the resistance of the fish to diseases is diminished. However, disease outbreaks are also related to fish overcrowding as well as the virulence of pathogens but is the synergy of pathogens and environmental stress that determine fish losses and mortality (FAO, 1993).

Pollution is considered an anthropogenic factor where human activities can affect fish life in sea and coastal waters. Intensive agriculture, industrial growth, urbanization and tourisms have severe impact in water pollution (Ziemann *et al.*, 1992). Chemicals contaminants like pesticides, organochlorines (Dethlefsen *et al.*, 1996), dioxin (Guiney *et al.*, 1996), DDT, toxins from algal blooms collapse (Noga *et al.*, 1996) and heavy metals can affect drastically fish health directly or trough their food even in low concentrations (Authman *et al.*, 2015). Furthermore biological degradation of

discharged organic material usually modifies the aquatic ecosystem where untreated waste may spread pathogens causing concern in fish and human health (Dudley *et al.*, 1980).

Also imbalances in water quality (temperature, pH, salinity, oxygen etc.) can cause dangerous growth of algae and plants that may determine an enhanced mortality in fish (FAO, 1993).

Several studies have shown that pollution can stress fish causing a reduction in the level of unspecific immunity possibly leading fish to immunosuppression that increases their susceptibility to pathogens (Snieszko, 1973). Specific contaminants (e.g. phenols, metals, pesticides etc.) are reported to determine a decrease in the number of leucocytes and antibodies often resulting in a wide array of immunosuppressive effects (McLeay & Gordon, 1977; Thuvander, 1989; Arkoosh *et al.*, 1998).

In the aquatic ecosystem, pathogens live in water, sediment, plants and animals and are responsible for diseases in wild and farmed fish (Obasohan *et al.*, 2010). Fish are reservoir of primary pathogens but they can also get opportunist invaders from environment. Virus bacteria and parasites use different way to determine infection in fish entering trough mouth, gills, ulcerated skin and gastrointestinal tract (Inglis *et al.*, 1994).

Recently environmentalists are increasingly afraid about the growth of pathogenic microbes due to organic wastes discharged into water. Essentially coming from animal husbandry operations, the high concentrations of bacteria and nitrates are becoming a serious environmental and human health concerns. Materials deposited by waters form sediment that can harbour pathogens and toxic substances threatening the fish health through ingestion of contaminated food (Obasohan *et al.*, 2010). Pathogens such as *Salmonella*, *Shigella*, *Aeromonas*, *Escherichia coli*, *Vibrio*, *Mycobacterium* spp., viruses and hookworm larvae have been reported associated to pollution and to reduced water quality. Some examples of environmental changes predisposing disease are shown in Table 1.5.

Table 1.5 Environmental factors and fish susceptibility to certain diseases (Wedemeyer & McLeay, 1981).

Disease	Environmental stress factors predisposing to disease
Furunculosis *(Aeromonas salmonicida)*	Low oxygen (≈ 4 mg l^{-1}); crowding; handling in the presence of *A. salmonicida;* handling for up to a month prior to an expected epizootic
Bacterial gill disease *(Myxobacteria spp.)*	Crowding; unfavourable environmental conditions such as chronic low oxygen (4 mg l^{-1}); elevated ammonia (0.02 mg l^{-1} unionized); particulate matter in water
Columnaris *(Flexibacter columnaris)*	Crowding or handling during warm (15°C) water periods if carrier fish are present in the water supply; temperature increase to about 30°C, if the pathogen is present, even if not crowded or handled
Kidney disease *(Renibacterium salmoninarum)*	Water hardness less than about 100 mg l^{-1} (as $CaCo_3$); diets containing corn gluten or of less than about 30% moisture
Hemorrhagic septicemia *(Aeromonas* and *Pseudomonas)*	Pre-existing protozoan infestations such as *Costia, Trichodina;* inadequate cleaning leading to increased bacterial load in water; particulate matter in water; handling; low oxygen; chronic sublethal exposure to heavy metals, pesticides or polychlorinated biphenyls (PCBs); for carp, handling after overwintering at low temperatures
Vibriosis *(Vibrio anguillarum)*	Handling; dissolved oxygen lower than about 6 mg l^{-1}, especially at water temperatures of 10–15°C; brackish water, of 10–15 per mille salinity
Parasite infestations *(Costia, Trichodina, Hexamita)*	Overcrowding of fry and fingerlings; low oxygen excessive size variation among fish in ponds
Spring viremia of carp and tail rot	Handling after overwintering at low temperatures. Crowding; improper temperatures; nutritional imbalances; chronic sublethal exposure to PCBs; or to suspended solids at 200–300 mg l^{-1}
Coagulated yolk of eggs	Rough handling; malachite green containing and fry more than 0.08% zinc, gas supersaturation of 103% or more; mineral deficiency in incubation water
"Hauling loss" (delayed mortality)	Hauling, stocking, handling in soft water (less than 100 mg l^{-1} total hardness); mineral additions not used; CO_2 above 20 mg l^{-1}
Blue sac disease of eggs	Crowding; accumulation of nitrogenous metabolic wastes due to inadequate flow patterns

1.7.1 Mugilids as biological indicator of water pollution

Costal waters are natural environments for mullets as they function as the connection between brackish water and sea. Thus, mullets living in estuaries located in area with high level of urbanization are constantly exposed to the action of increased quantity of chemical and other contaminants discharged in water (Kennish, 2002). These pollutants (e.g. metals, pesticides, surfactants etc.), have multifarious effects on Mugilid health and the consequences affect mullets directly or via secondary outcomes like reduction of natural food that lead to a diminished resistance to disease (FAO, 1993). Through the

collection and analysis of samples mullets could be used as an indicator of the actual state of the environment. Thus, according to the National academy of sciences-national research council (NRC, 1991) definition, these species can be proposed as a biological sentinel to detect the degree of pollution of the marine ecosystems. The animals used to regulate and monitor the environment are called "animal sentinel system" (SSA) and identify a wide variety of environmental pollutants hazardous to human, animal health and ecosystems. The benthic feeding permit to incorporate heavy metals and other pollutants giving rise to a bioaccumulation of these contaminants in mullet tissues. Gills are always considered the primary target organ of the contaminants and toxins (Fernandes *et al.*, 2003; Pereira *et al.*, 2010). The different species of mugilids show a wide range in the sensitivity to different pollutants: juvenile *Liza ramada* are susceptible to herbicides but at low concentrations liver can still recovery its degenerative state (Biagianti-Risbourg & Bastide, 1995). Heavy metals are detected in *Liza ramada* in the Tagus Estuary, Portugal, and are related to their feeding habits that prefer finer sediment fractions (Pedro *et al.*, 2008). Moreover the same mullet specie shows an increased polychlorinated biphenyls (PCBs) level with age (Baptista *et al.*, 2013). Analogously, the increase of mercury in the tissues of *Liza aurata* measured in two different coastal ecosystems revealed a higher mercury concentration in the most polluted area (Tavares *et al.*, 2011); moreover the gills has been used as a probe of a gradient of pollution in the Óbidos Lagoon, Portugal (Pereira *et al.*, 2010).

From the above remarks Mugilides can act as a sentinel system and can be used to monitor the level of pollution and its distribution in the environment in order to reduce the risk assessment for the man and for the animal population and to check water quality in area where urban pressure near the coast can affect extensive aquaculture system. As a further remark in Italy tourism is one of the activities that have a major impact on these environments. It is the case of lagoons such as Lesina or Orbetello or of many Sardinian ponds (FAO, 2015a).

1.8 Pathogens in mullets

Diseases are caused by primary or opportunist pathogens in both wild and cultured fish. Many of these pathogens do not cause directly disease in fish but when encountering an immunocompromised host they could determine infections (Richards & Roberts, 1978). Infectious organisms are generally found as common component of the water flora mainly in eutrophic environments where stressful condition predisposes fish to bacterial

diseases (Snieszko, 1974).

A significantly role in culturing mullet is played by infectious diseases and parasites where disease-outbreaks have determined one of the main obstacles in the development of aquaculture industry. Several species of parasites can infect mullets: protozoa (*Myxobolus episquamalis* and *Myxosporidium mugilis*), trematodes (*Microcotyle mugilis*, *Tetraonchus vanbenedenii* and *Bedenia monticellii*), crustaceans and copepods (*Ergasilus nanus*, *Caligus bonito, Lernanthropus mugilis, Lernaenicus neglectus* and *Branchiella oblonga*) (Kim *et al.*, 2013). Viral and fungal diseases were also observed in farmed mullets. Most of the known fungal pathogens are associated with genera *Aphanomyces*, *Achlya*, *Phialemonium*, *Ichthyophonus* sp.

Virus disease are usually related to high mortality (lymphocystis; viral nervous necrosis; piscine Nodavirus infection; infectious pancreatic necrosis) (Ovcharenko, 2015). Farmed mullet are more susceptible to bacterial pathogens (*Photobacterium damselae* subsp. *piscicida*, ex *Pasteurella piscicida*, *Streptococcus aquamarinus*, *Achromobacter*, *Escherichia intermediate*, *Aeromonas hydrophila A. salmonicida*, *Vibrio anguillarum*) that may persist for a long time on sediments containing decaying organic matter. Polluted water can induce stress in fish inviting secondary infection. Mullets can act as vectors of dangerous bacteria transmissible to human posing a real problem for public health (*Aeromonas hydrophila*, *Mycobacterium marinum*, *M. fortuitum*, *Vibrio parahaemolyticus*, *Erysipelothrix rhusiopathiae* and *Leptospira icterohaemorhagiae*) (Paperna & Overstreet, 1981).

1.8.1 Atypical mycobacteria

Several bacteria belonging to the genus *Mycobacterium* are characterized by a specific acid-alcohol resistance properties; this characteristic is due to the particular structure of their cell wall mainly composed of mycolic acids. Mycobacteria are nonmotile, aerobic, nonsporulating and rod shaped bacilli and considered as Gram-positive, even if, for the particular composition of their cell wall, this type of staining is not suitable for their identification. Their detection is best obtained using the Ziehl-Neelsen (ZN) method where mycobacteria are stained in red (Ziehl, 1882; Neelsen, 1883).

The classification of mycobacteria is very complex and this group include pathogenic mycobacteria causing tuberculosis (*Mycobacterium tuberculosis*, *M. africanum*, *M. bovis*, *M. microti*). Atypical mycobacteria (AM), known also as mycobacteria other than tuberculosis (MOTT) include several species of the genus *Mycobacterium* and they are

able to produce progressive chronic diseases in mammals, birds, reptiles and fishes. They are considered different from those of the *Mycobacterium tuberculosis* complex and *M. leprae* by the fact that they are not obligate pathogens but are opportunistic inhabitants of the environment (Primm *et al.*, 2004).

In 1959 Runyon categorized atypical mycobacteria into 4 groups according to the pigmentation, the morphology and the growth speed of the colonies including both slow-growing (i.e., colony formation requires 7 days or more) and rapidly growing species (i.e., colony formation in less than 7 days).

In the past different classifications have been proposed to classify mycobacteria. (Bojalil *et al.*, 1962; Woods & Washington, 1987) The current one distinguishes the mycobacteria species based on their clinical and pathological findings (Rastogi *et al.*, 2001) and can be summarize as follow:

- *Mycobacterium tuberculosis complex* (MTC): *M. tuberculosis, M. bovis, M. africanum, M. microti, M. canettii, M. bovis* subsp. *caprae, M. pinnipedii*
- *Mycobacterium avium complex* (MAC): *M. avium* subsp. *avium, M. avium* subsp. *paratuberculosis, M. intracellulare, M. hominisuis*
- *Mycobacterium other than tuberculosis complex* (MOTT): includes numerous species ubiquitous in nature, of which only *M. leprae* is an obligate pathogen.

1.8.2 Mycobacteriosis in fish

The term “mycobacteriosis” or “fish tuberculosis” is a chronic systemic and progressive disease caused by mycobacteria belonging to the genus *Mycobacterium*. About 150 species of fish are susceptible to infection of mycobacteria and have been found in ornamental and farmed fish also devoted to human’s consumption (Nigrelli & Vogel, 1963). Mycobacteriosis in fish is characterized by the presence of whitish gray nodules (granulomas) scattered both at cutaneous level and in visceral organs (Ghittino, 1985; Zanoni *et al.*, 2008). The mycobacteria species most commonly found both in marine and fresh water as well as in wild, farmed and ornamental fish are mainly represented by *M. marinum*, *M. fortuitum*, *M. chelonae* (Adams *et al.*, 1970; Wolke & Stroud, 1978; Noga, 1996) and less commonly by *M. terrae, M. gordonae, M. scrofulaceum, M. simiae, M. montefiorense, M. shottsii, M. chesapeaki, M. poriferae, M. neoaurum* (Lansdell *et al.*, 1993; Tortoli *et al.*, 1996; Herbst *et al.*, 2001).

The mycobacteria were isolated from the environment and from cold-blooded animals in all parts of the world. Their distribution is due to their aptitude to grow at different

temperatures and to survive and proliferate in different environments (stagnant water, mud, rivers, lakes, drinking water, swimming pools). Soil and water are so considered as a *reservoir* of saprophytes mycobacteria where they can remain viable for months or years (Goslee & Wolinsky, 1976), able to survive in unfavourable environmental conditions (Kim & Kubica, 1973). The mycobacterial species most found in the environment are *M. gordonae*, *M. kansasii*, *M. terrae*, *M. phlei*, *M. fortuitum*, *M. chelonae*, *M. scrofulaceum* (Biondi *et al.*, 1982). The first cases of mycobacteriosis in fish have been reported by several authors and are mainly caused by three species of the genus *Mycobacterium* (*M. chelonae, M. fortuitum* and *M. marinum*) (Gauthier & Rhodes, 2009). *Mycobacterium marinum* is the most commonly encountered bacteria in salt water fish (Jacobs *et al.*, 2009). In contrast, *M. chelonae* was identified in cold-water fish, especially in salmonids (Brocklebank *et al.*, 2003). Recently, cases of infection from other forms of mycobacteria as *M. gordonae* and *M. terrae* have been reported in fish even destined for human consumption (Mediel *et al*., 2000; Varello *et al.*, 2007).

The list of mycobacteria is constantly increasing due to the new molecular characterization systems used for their identification. Recently it has been described the first isolation of *Mycobacterium salmoniphilum* and *Mycobacterium chelonae* in sturgeons (*Acipenser gueldenstaedtii*) from a fish farms in Italy (Antuofermo *et al.,* 2014; Righetti *et al.*, 2014). Although various routes of infection are possible in fish, oral transmission through feces or carcasses of infected fish are recognized as the primary cause of mycobacteriosis (Ackleh *et al.*, 2014). Furthemore, it has been demonstrated that amoebae or *Paramecium caudatum* are usually required for efficient transmission of *M. marinum* in some species (e.g. zebrafish: Harriff *et al.*, 2007; Peterson *et al.*, 2013). Other possibilities are equally plausible, including entrance through dermal wounds and transovarian transmission has been documented in fighting fish (*Betta splendens*) (Chinabut *et al.*, 1994).

Most of experimental infections have used the intraperitoneal and intramuscular routes (Wolf & Smith, 1999; Gauthier *et al.*, 2003). Moreover zebrafish embryos have been infected with *M. marinum* via bath exposure (Davis *et al.*, 2002), as well as in adult zebrafish infected with *M. marinum* and *M. peregrinum* the gastrointestinal tract was indicated as the primary route of infection (Hariff *et al.*, 2007). Nevertheless, mycobacteriosis seems to occur in association with seasonal factors including the availability of food, quality and water temperature as well as population density (Bragg

et al., 1990; Hennigan *et al.*, 2013). Essentially the diagnosis of mycobacteriosis is based on the detection of granulomas and acid-fast bacteria in tissue sections that are visible with Ziehl-Neelsen stain (Gauthier & Rhodes, 2009). However the diagnosis of fish mycobacteriosis is very complex and several investigations are necessary to identify mycobateria. Thus, histopathology remains the most common method for the diagnosis of fish mycobacteriosis, whereas culture and PCR-based methods are required for species identification (Toranzo *et al.*, 2005). Normally symptoms are nonspecific (uncoordinated swimming, anorexia, apathy, weight loss, exophthalmos and ascites) and can be easily confused with other bacterial or fungal infections. The period of incubation of mycobacteriosis is not well established, and the first symptoms may arise from 6 weeks to 2 years based on the host's immune system and water temperature (Ashburner, 1977).

The disease may take several years to progress from the asymptomatic state to clinical illness (Snieszko, 1978; Bragg *et al.*, 1990; Noga *et al.*, 1990). Grossly, lesions caused by mycobacteria are gray-whitish nodules of varying diameter, disseminated in all visceral organs and in particular in the spleen, liver, kidney and gonads (Fig 1.11). Nodules can also be observed in the gills between lamellae (Gauthier & Rhodes, 2009).

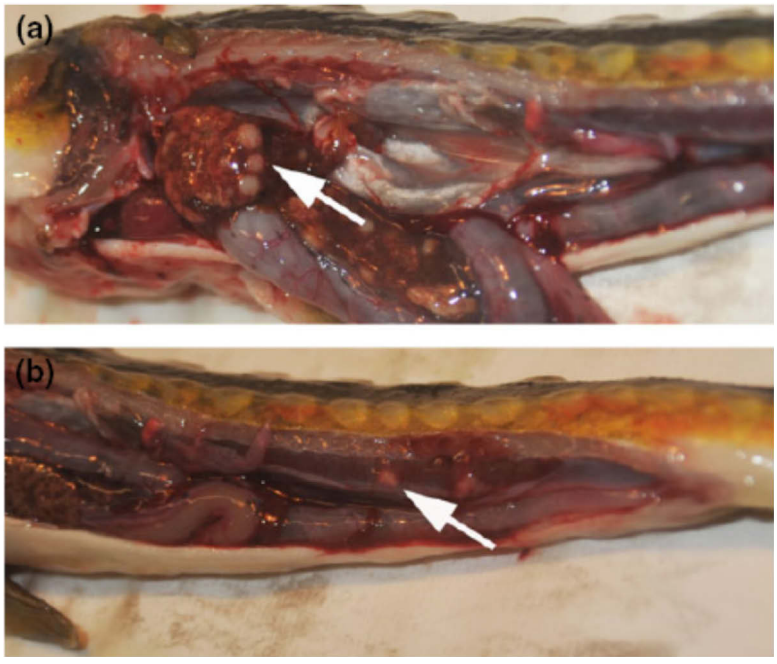

Fig. 1.11 Visceral organs of a Russian sturgeon infected by *Mycobacterium salmoniphilum*. (a) Multiple white roundish nodules are clearly visible in the liver (white arrow). (b) Irregular nodules in shape are dispersed throughout kidney (white arrow) (photo by Righetti *et al.*, 2014).

Microscopically atypical mycobacteria are pleomorphic rods of 1.5 X 0.25 micron in size. Typical granulomas are composed of epithelioid cells surrounded by a connective capsule of varying thickness, often associated with an area of central necrosis with a variable amount of acid-fast bacilli evident with Ziehl-Neelsen stain (Astrofsky *et al.*, 2000; Decostere *et al.*, 2004) (Fig 1.12).

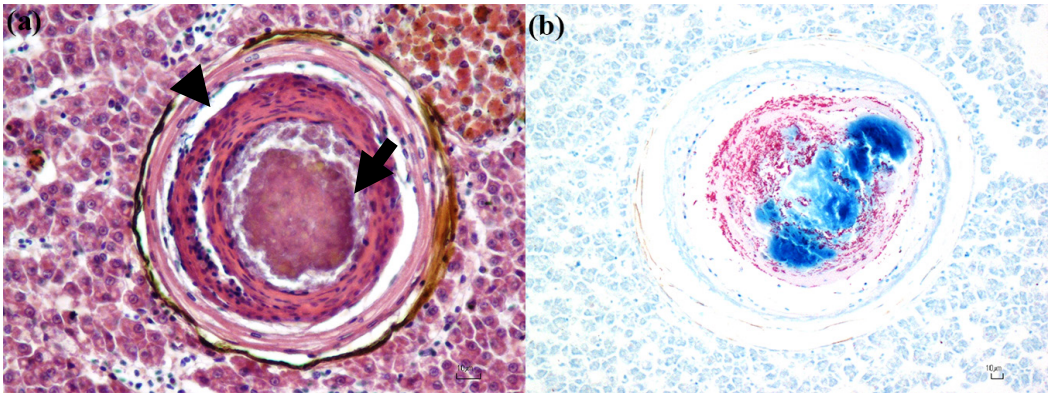

Fig 1.12 Microscopic aspect of granuloma due to atypical mycobacteria in fish. (a) Liver: granulomas are composed of epithelioid cells (arrow head) surrounded by a connective capsule often associated with an area of central necrosis (arrow). (b) Granuloma with fast rod shape bacilli positive to Ziehl-Neelsen.

Furthermore, as experimentally observed by Colorni *et al.* (1998) and recently by Ortega *et al.* (2014) and Antuofermo *et al.* (2016) granulomas can be also histologically classified in distinct evolutionary stages.

According to the histological pattern granulomas can be differentiated in relation to the disease development and they can be classified as subacute and chronic granulomas. Firstly granuloma is represented by an infiltration of flat epithelioid cells surrounding necrotic foci within it is possible to detect the presence of alcohol-acid resistant bacilli. Later evolutionary stage granulomas are characterized by the presence of a central portion of coagulative necrosis where there are colonies of mycobacteria, surrounded by epithelioid cells and by more concentric layers of fibroblasts. Chronic granulomas not always allow the identification of bacterial colonies. For a final diagnosis of mycobacteriosis, however, isolation and identification of the microorganism species are necessary since genus of mycobacteria is highly heterogeneous in terms of epidemiology and pathogenicity.

At this scope, specific media and phenotypical tests are commonly used. The classical method still used today is based on the phenotypic approach that unlikely takes a long

time (several weeks are required to mycobacteria for growth and achievement of their metabolic activity, which is essential to address the biochemical tests), and does not always produce a precise identification (Kent & Kubica, 1985). The cultural examination for the detection of mycobacteria, prepared on specific solid media (Lowenstein-Jensen and Stonebrink) are evaluated for morphology, temperature (28, 37, 43°C), speed of growth, and the ability to produce pigment (Fig. 1.13).

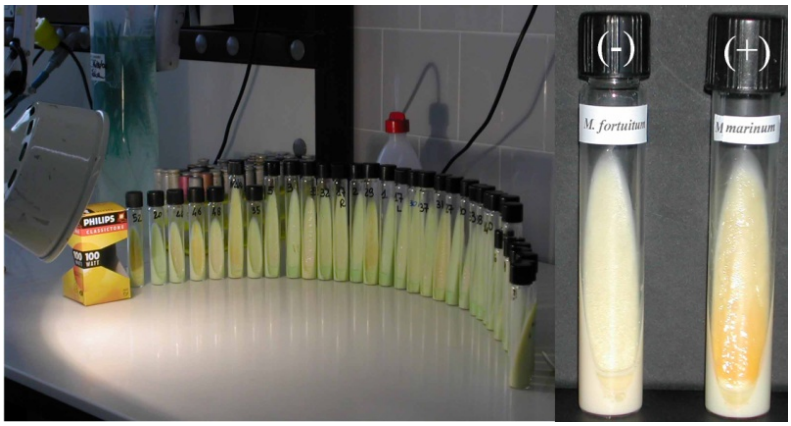

Fig. 1.13 Photo-induction show chromogenic colonies of *M. marinum* (right tube) (photo by Florio *et al.*, 2003).

These classical methods, however, are not always conclusive to identify all mycobacteria species. The culture condition can influence morphology and biosynthesis and sometimes the presence of strain varieties can alter the typical biochemical profile of mycobacteria (Ucko *et al.*, 2002 and references therein). *Mycobacterium marinum* is one of the main fish pathogen described in association to zoonotic disease, thus the ability to distinguish among different strains remains of great importance (Haenen *et al.*, 2013). So, different molecular techniques based on specific markers amplification and sequencing has been developed (Srinivasan *et al.*, 2014).

In particular, the sequence comparison between stable parts of the bacterial genetic code allow at the same time bacterial identification and phylogenetic classification (Clarridge, 2004). For this reason, sequence analysis of the 16S rRNA gene has been widely used over the last two decades to establish phylogenetic relationships of bacteria, opening the way to molecular epidemiologic analysis. As previously reported PCR amplification and direct sequencing of 16S rRNA products allows proper taxonomic assignment in Mycobacterium species. (Ucko *et al.*, 2002 and references therein). The 16S rRNA gene is a stable part of the bacterial genome composed by both

conserved and variable sequence regions (Srinivasan *et al.*, 2015). The conserved regions sequences represent an useful tool to establish bacterial taxonomic levels, while the considerable sequence diversity in the 16S rRNA variable regions represents the most important current target for species classification (Chakravorty *et al.*, 2007; Větrovský & Baldrian, 2013). However, the wealth of data available from public sequence databases highlighted that the comparison of the 16S rRNA sequence is often insufficient in closely related species (Ucko *et al.*, 2002 and references therein).

For this reason, the protein-encoding genes analysis may be more discriminative than those encoding rRNA, and the highly conserved hsp65 gene of *Mycobacterium* species represents a useful candidate for this purpose, showing species-specific variations sequence (Ucko *et al.*, 2002 and references therein). Nevertheless, hsp65 gene reveals higher sequence variability within the genus. However mycobacteria species are numerous and in order to identify species restriction enzyme digestion of PCR products by BstEII and HaeIII and separation of the restriction fragments on an agarose gel is required. Sequencing restriction pattern allow identifying different mycobacterial species (Telenti *et al.*, 1993; Toranzo *et al.*, 2005).

Prophylaxis is the best measure to prevent the transmission of atypical mycobacteria in fish due to the high resistance of these microorganisms in the environment. Formalin, sodium hypochlorite or phenolic compounds are necessary for the disinfection of the tanks in which there has been an infection with mycobacteria (Thoen & Schliesser, 1984). Additionally it is important to reduce overcrowding in fish farming or aquarium and immediately remove sick and dead fish. Also any importation of new individuals should be subjected to a quarantine period of 4-8 weeks (Belas *et al.*, 1995). Treatments of affected individuals are not recommendable because atypical mycobacteria are resistant to the common chemotherapeutic agents used to treat human tuberculosis (Conroy & Solarolo, 1965).

All fish must be considered susceptible to mycobacteriosis, with a higher prevalence in farmed fish, where there may be significant losses (Gauthier & Rhodes, 2009). Anyway mortality in farmed fish is never on a large scale, but it is usually expressed in outbreaks (Gauthier *et al.*, 2008). The spreading of the disease may be related to improper farm management that can induce a decrease in the resistance of the fish (Chinabut, 1999). Some fish species are still poorly studied and mycobacteriosis descriptions in mugilidi, are reported in the literature only sporadically. At present, acid-fast bacterial infections in wild Mugilidae are scarcely reported worldwide: Osman (1980) observed several

cases of mycobacteriosis in *Liza aurata* from Libya; Couch (1985) in an adult of *Mugil cephalus* from the Gulf of Mexico. A recent study revealed in Venezuela the presence of alcohol acid bacteria in 25% in *Mugil curema* (Aldeima *et al.*, 2001). In Italy mycobacteriosis was detected in 2011 in mullets of the coastal lagoon in Lake Faro (Messina) and in 2014 in the eastern coast of the Ligurian Sea (Marino *et al.*, 2012; Varello *et al.*, 2014).

Furthermore, fish mycobacteriosis was also detected in cultured mullets (Aldeima *et al*., 2001; Salati *et al.*, 2010). Among the emerging diseases of bacterial origin, fish mycobacteriosis are still poorly studied in Sardinia, one of the Italian regions most devoted to extensive aquaculture activities with more than 12.000 hectares of ponds and coastal lagoons.

1.8.3 Public health

Fish mycobacteriosis is a concern for public health because atypical mycobacteria act as zoonotic agents. In humans, skin infections by non-tuberculous mycobacteria are uncommon, but their importance has changed over the past years (Giavenni, 1980; Ghittino, 1985; Ghittino & Bozzetta, 1994; Zanoni *et al.*, 2008) and has reached epidemic proportions (Nichols *et al.,* 2004). *Mycobacterium marinum* has been recognized as the causative agent of "swimming pool granuloma" and it was isolated for the first time from skin lesions in swimmers of a thermal pool in Sweden (Norden & Linell, 1951).

In humans it causes skin granulomatous inflammation at the extremities (hands and fingers) in areas with pre-existing injuries (Philipott *et al.*, 1963; Adams *et al.*, 1970; Lim *et al.*, 2000; Lahey, 2003). Skin lesions including dermal abscesses, fistulas, suppurative granulomas or multifocal nodules with sporotrichoid eruptions. Lesion can be firstly focal of red bluish colour with a diameter of 5-6 centimetres and evolving into skin ulceration. This disease in human is known as fish tuberculosis, piscine tuberculosis, fish tank granuloma, or possibly swimming pool nodules generally caused by *Mycobacterium marinum* (Fig. 1.14).

Mycobacterium marinum has an optimum growth temperature of 30°C, and does not grow well at 37°C so is often missed in hospital laboratories. Two other species of mycobacteria *M. fortuitum* and *M. chelonae* are considered as zoonotic agents. These are considered as opportunistic pathogens causing generally skin infections but also lung abscess, endocarditis, meningitis and osteomyelitis (McCracken *et al.*, 2000).

Disease caused by atypical mycobacteria develops mainly in immunocompromised patients like in HIV-positive patients (Roson *et al.*, 2002; Satta *et al.*, 2002).

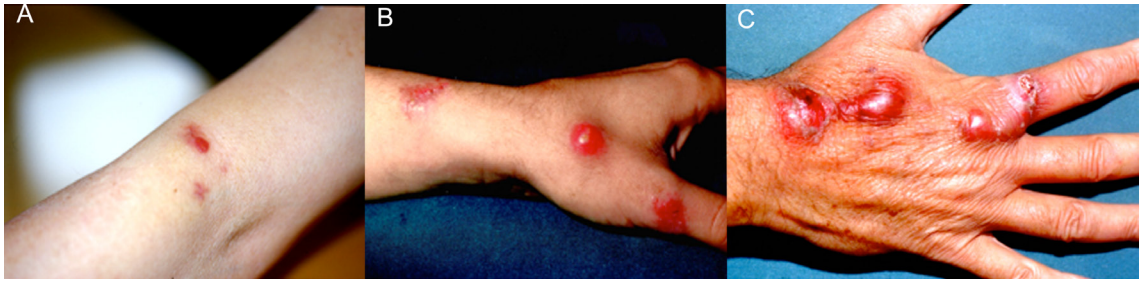

Fig. 1.14 Macroscopical aspect of tank granuloma in human caused by atypical mycobacteria. (A) Elbow haired skin: focal, granulomatous mild dermatitis. (B) Forearm and left hand haired skin: multifocal, granulomatous moderate dermatitis. (C) Right hand haired skin: multifocal, granulomatous to necrotizing severe dermatitis (photo by Marino Prearo).

Nontuberculous mycobacteria have been repeatedly isolated from lung infections and they have radiographical and histological findings indistinguishable from those caused by *M. tuberculosis* (Thorel, 1994). For a proper diagnosis a history that tells exposure to fish, swimming pools, natural water basins, is important in patients with skin infections. Even the treatment of such conditions is difficult due to the inefficacy of human tuberculosis therapy against atypical mycobacteria (Terry *et al.*, 2001).

Despite the disease usually has a favourable outcome, in a study by Wallace it turned out that outbreak caused by atypical mycobacteria was fatal in 9% of a group of 125 patients (Wallace *et al.*, 1998). It should be emphasized that in recent years infections due to MOTT increased in industrialized countries (Thorel, 1994). This disease is considered a real risk for operators and has been frequently described in fishermen and aquarists (Petrini, 2006).

At local scale, 18 human granulomatous skin lesions, mainly located at the extremities, have been diagnosed as tank granulomas in Sassari Hospitals from 2006 to 2016 (Table 1.6). As a result of these considerations it is of great importance increase the prophylaxis and improve information in the most at-risk sectors, such as fish markets and processing industries.

Table 1.6 Cases of tank granuloma diagnosed in Sassari Hospitals from 2006 to 2016.

ID	Year of diagnosis	Job	Age/year	Anatomical localization
1	2006	bricklayer	68	wrist hand right
2	2006	operator	65	hand
3	2006	*na*	45	right hand
4	2006	maritime	83	back hand
5	2006	*na*	58	left hand III finger
6	2007	student	38	forearm
7	2009	worker	37	back hand left
8	2011	office worker	63	right elbow
9	2011	municipal inspector	58	right hand back
10	2011	municipal inspector	58	forearm
11	2012	retired - carpenter	72	right arm
12	2013	retired	73	left arm
13	2013	retired	77	right hand
14	2013	merchant	48	hand back
15	2013	student	17	left forearm
16	2014	breeder	73	right forearm
17	2014	housewife	64	right hand back
18	2016	mechanic	45	right hand III finger

na: not available

1.9 Endocrine Disruptors Compounds (EDCs)

In recent decades, awareness of the importance of environmental and terrestrial ecosystems protection has become a priority. There has been an increased interest from the international scientific community regarding the environmental contamination from natural or synthetic substances and the effects that these agents could exert both on population and environment. In the past two decades many studies have highlighted that environmental contaminants can interfere with the endocrine system causing negative effects on human and animal health (Diamanti-Kandarakis *et al.*, 2009). Some of the more damaging chemical contaminants are classified as endocrine disrupting chemicals (EDCs) and they have been widely found in the river and sea water due to their release by effluents from sewage treatment plants of urban waste water, agricultural and livestock activities (WHO & UNEP, 2013).

An EDC was defined by the U.S. Environmental Protection Agency (EPA) as: “an

exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Kavlock *et al.*, 1996). The EDCs includes both inorganic and organic compounds that may be of natural and synthetic origin, such as organochlorine, pesticides, herbicides, fungicides, plastics, surfactants, cosmetics, dioxin, alkylphenols (APs), polychlorinated biphenyls (PCBs), heavy metals and pharmaceuticals such as synthetic estrogen like 17α-ethinyl estradiol (EE2) also used as a contraceptive. Among the natural EDCs we find steroidal estrogens derived from animal and human metabolism, phytoestrogens and mycoestrogens (Goksøyr *et al.*, 2003; WHO & UNEP, 2013). Exposure to EDCs can cause complex biological effects in aquatic organisms such as changes in behaviour, growth, development, metabolism and reproduction. Such effects have been observed in a wide variety of animals such as birds (Fry, 1995), amphibians (Palmer & Palmer, 1995) and fish (Sumpter & Jobling, 1995; Allen *et al.*, 1999; Pait & Nelson, 2002). The fish are particularly sensitive to the action of the EDCs and some fish species are able to bioaccumulate and or biomagnify some of these compounds, thus representing a further hazard to human health. By virtue of these features, Teleosts have been used in numerous studies as sentinel organisms to monitor pollution (Van der Oost *et al.*, 2003). EDCs can affect also fish reproduction, gamete quality and progeny through direct effects on the hypothalamic-pituitary-thyroid and/or hypothalamic-pituitary-gonadal axis (Schreck *et al.*, 2001; Hachfi *et al.*, 2012).

The molecular structure of EDCs does not allow identifying a common mechanism of action of all these compounds and today research shows that the ECDs pathways are much broader than originally recognized (Diamanti-Kandarakis *et al.*, 2009). Of the more than 900 chemicals identified as confirmed or potential endocrine disruptors, more than 200 seem to possess estrogenic activity. Depending on the similarity of EDCs and natural hormones molecular structures according to Goksøyr *et al.* (2003), these are capable of:

1. mimic the effects of natural sex steroid hormones (estrogens and androgens) by binding to hormone receptor or influencing of cell signal transduction;
2. hinder the binding of a steroid hormone to its receptor influencing the cell signal transduction;
3. alter the synthesis, transport, secretion and degradation of natural hormones;
4. change the production and/or function of hormone receptors.

Sex steroids, androgens in males and estrogen in females, play a major role in sexual differentiation, development and reproductive behavior of teleost fish. Some of the most powerful EDCs like some natural and synthetic estrogens (EE2) act as receptor agonists when they bind to the steroids receptors Most of the EDCs possess a lower affinity if compared to endogenous sex steroids, however, often being ubiquitous in the aquatic environment are a source of continuous exposure to wild fish populations. Furthermore androgenic activity has been proven for a limited number of chemical compounds while scarce data are available on the interaction of EDC with progesterone receptors (Söffker & Tyler, 2012). Numerous studies using fish as experimental models (eg. *Danio rerio*) has shown a wide range of effects caused by estrogen, androgen as well as by EDCs. Estrogen and androgen antagonists, although they have two different mechanism of action, show some similar effects on the reproductive system of fish. These hormones are able to determine feminization phenomenon (Nimrod & Benson, 1998*;* Parrott & Blunt, 2005*;* Seki *et al.*, 2005; Kang *et al.*, 2006; Larsen *et al.*, 2008), to reduce or inhibit spermatogenesis (Jobling *et al.*, 1996*;* Kiparissis et *al.*, 2003*;* Jensen *et al.*, 2004) and to delay the ovarian maturation (Van den Belt *et al.*, 2002; Kiparissis *et al.*, 2003; Weber *et al.*, 2003). Only estrogens, however, possess the ability to induce the production of vitellogenin (VTG) in male subjects (Jobling *et al.*, 1996; Versonnen & Janssen, 2004). On the other hand androgens and estrogen antagonists can induce masculinization and determine an increased testicular size (Örn *et al.*, 2006) with stimulation of spermatogenesis. Both have the ability to reduce or inhibit ovulation and the production of vitellogenin and yolk proteins in females (Ankley *et al.*, 2009). In addition, they are able to change the sex ratio to males (Örn *et al.*, 2003; 2006; Hahlbeck *et al.*, 2004) and reduction in size of the ovary in females (Pawlowski *et al.*, 2004; Seki *et al.*, 2005). As remarked above xenoestrogens in fish induce vitellogenesis in male individuals. Thus, EDCs determine an increase VTG levels in males after exposure to their actions as observed in several species, including rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), chub (*Leuciscus cephalus*) and other fish species (WHO & UNEP, 2013). So the vitellogenin, is commonly measured in environmental monitoring programs and used as an indicator of exposure to estrogen. It is a phospholipoglycoprotein that is synthesized in the liver of females and transported from plasma to the receptors that transport VTG into oocytes where it is used for the synthesis of the yolk protein, essential nutrient and energy source for the embryo (knows as vitellogenesis). The male fish normally synthesize very low

quantities of VTG, since the gene coding for the protein is usually silent in male subjects. The vitellogenesis is a process related to the levels of estradiol (Anderson *et al.*, 1996) and in females VTG is relatively high. The estrogen receptor (ER) regulates the synthesis of VTG by binding to estradiol, but also show the ability to bind estrogen analogs. Consequently xenoestrogens, through binding to the estrogen receptors, trigger a series of biological reactions in male subjects that culminate with the activation of the gene coding for the VTG. The presence of estrogenic compounds in the aquatic environment determines an increase in levels of VTG and a consequent process of feminization in males. Therefore studies reported that the effects on fish health caused by EDCs present in aquatic environments are to a large extent related to the phase of growth and development of the organisms. It has been shown that the fish seem to be more sensitive to EDCs in the juvenile stages, in particular immediately after hatching or before morphological sexual differentiation (Jobling *et al.*, 1998). Therefore exposure to EDCs causes serious reproductive problems in fish and to their progeny such as gonadal dysgenesis, reduced fertility, abnormal production of gametes, behavioural changes and other reproductive issues. In particular EDCs lead to a reduction of the volume and weight of the gonads and to disorders in their maturation, follicular atresia and especially an increase in the presence of intersex (Blazer *et al.*, 2007; Puy-Azurmendi *et al.*, 2013; Bizarro *et al.*, 2014). In fact, numerous studies depict a close association between the detection of intersex conditions and the presence of EDCs in marine and freshwater aquatic environments (Lavado *et al.*, 2004; Baldigo *et al.*, 2006; Woodling *et al.*, 2006; Blazer *et al.*, 2007; Vajda *et al.*, 2008). The presence of intersex conditions in fish is then considered as an indicator of exposure to EDCs.

1.9.1 Intersex in mullets

Intersex conditions in different wild populations of fish and amphibians have been frequently reported (Jobling *et al.*, 1998; Hinck *et al.*, 2006; Murphy *et al.*, 2006). Exposure to EDCs interfere with the development and functioning of the fish endocrine system, causing a large number of reproductive disorders, including reduction in gonad weight and volume, abnormalities in gonadal maturation, gonadal atresia, and especially an increased incidence of intersex (WHO & UNEP, 2013). The term intersex describes alterations in gonadal development with the simultaneous presence of male and female reproductive stages in the same gonad of a gonochoristic species. This condition has

been detected in several aquatic animals and interpreted as a signature effect of exposure to EDCs and indicates also the occurrence of testicular oocytes, testicular follicles, testis-ova or ovotestes (Hecker *et al.*, 2006; Bahamonde *et al.*, 2013). In particular, the presence of oocytes in the testes of adult or sub-adults males (i.e. the testicular oocytes, TOs) represents the most commonly reported intersex condition in fish (Abdel-Moneim *et al.*, 2015 and references therein). Different terminologies have been used to define the intersex condition, so it is often difficult to compare the results obtained by various authors to standardized terminology. Hecker *et al*. (2006)proposed a classification of gonadal abnormalities in testicular oocytes (TOs), ovotestis, mixed gonadal tissue and gonads segmented. The TOs in fish represent the most intersex event reported in the literature, described as the presence of oocytes (single or multiple) dispersed within the testes of adult or sub-adults males (Bahamonde *et al.*, 2013; Abdel-Moneim *et al.*, 2015). The term ovotestis has been used, over the years in different ways by different authors, often as a synonym of intersex. For some of them, this term has been defined as the presence of oocytes within the male gonads (Allen *et al.*, 1999), by others, it has been used to describe a gonad with female gonadal tissue in a percentage greater than 45% (Hecker *et al.*, 2006). Furthermore, Getsfrid *et al.* (2004) have made a distinction between ovotestis and testis-ova, respectively, indicating in the first case the presence of mature ovarian tissue containing multifocal areas of testicular tissue and the presence of mature testicular tissue mixed with scattered oocytes (testis-ova). In accordance with Hecker *et al.* (2006), ovotestis indicates that a gonad shows female gonadal tissue in a percentage higher than 30%. The condition of mixed gonadal tissue shows the simultaneous presence in the same individual of testicular and ovarian tissue and this abnormality may be unilateral, or bilateral if both gonads are affected. When an individual presents multiple gonads or gonads are segmented into discrete subunits separated by a thin layer of connective tissue, it is called segmented gonads.

1.9.2. Histological classifications of intersex

Intersex condition may manifest gonadal abnormalities of different severity, ranging from scarce cells to extensive areas of gonadal tissue of the opposite sex. Moreover, in most cases, the gonads appear normal and are not detectable macroscopic changes. To diagnose intersex therefore it is necessary to perform histological examination of the gonad. Microscopical evaluation is necessary to reveal oocytes within the testicular tissue and to assess the severity of this disorder (Pait *et al.*, 2002). Numerous studies

have tried to classify the gonadal abnormalities detected in fish male into different classes of intersex severity, from mild to severe, in relation to the number, maturity and the distribution of oocytes within the testicular tissue (Jobling *et al.*, 1998; Van der Ven *et al.*, 2003; Bateman *et al.*, 2004; Blazer *et al.*, 2007). In literature, the histological classification systems of intersex severity are numerous and are typically based on the assignment of numerical values to the observed microscopic fields. The main microscopic evaluation systems of intersex severity in relation to different fish species are reported in Table 1.6.

1.9.3 Distribution of intersex

Before the '90s, intersex condition was rarely described just in wild freshwater fish (Jafri *et al.*, 1979; Slooff *et al.*, 1982; Blachuta *et al.*, 1991). In recent decades, however, the growing awareness of the presence of EDCs in the environment encouraged scientific researchers to study the effects of these compounds on vertebrate and invertebrate organisms. Recently governments and organizations like the World Health Organisation (WHO) have sponsored studies on the occurrence of intersex in wild fish founding environmental monitoring campaigns. A high incidence of intersex was detected, for example, in the wild populations of roach (*Rutilus rutilus*) living near the wastewater treatment plants (Jobling *et al.*, 1998). In estuarine and marine fish the intersex condition was observed in flounder (*Platichthys flesus*) and eelpout (*Zoarces viviparus*), proving that the action of EDCs extends beyond the internal river systems, reaching coastal waters (Allen *et al.*, 1999; Simpson *et al.*, 2000; Stentiford *et al.*, 2003). The largest number of intersex in fish has been reported in North America and Europe especially in the UK. With regards to such a geographical distribution it could be related to the presence of a greater number of researchers studying the phenomenon, rather than a real increased incidence of intersex in these sites. The Cyprinidae and Centrarchidae families show the higher percentage of intersex (26% and 18% respectively); the smallmouth bass (*Micropterus dolomieu*) is the most commonly cited fish species (approximately 10%), followed by the roach (*Rutilus rutilus*) and the largemouth bass *Micropterus salmoides* (Abdel- Moneim *et al.*, 2015).

An important point to note is that not all gonochoristic species develop intersex condition, even if they live in contaminated environments. Almost certainly some families are more capable than others to develop gonadal disorders.

Table 1.7 Classification and histological evaluation of the severity of intersex.

Authors	*Methods of assessment of the severity intersex*	*Species*
Van Aerle *et al.,* 2001	**A** = ≤ 5 primary oocytes for section **B** = 5 primary oocytes for section **C** = primary and secondary oocytes >50%	*Gobio gobio*
Anderson *et al.,* 2003	**Score 1=** immature oocytes <10 in 20 field **Score 2**= 10-20 immature oocytes in 20 field **Score 3**= oocytes >20 in 20 field	*Micropterus*
Jobling *et al.*, 1998	**Score 0**= normal testis **Score 1**= presence of spermatic duct, ovarian cavity and rare primary oocytes **Score 2**= presence of spermatic duct, ovarian cavity and infrequent primary oocytes **Score 3**= presence of spermatic duct, ovarian cavity and several primary oocytes often in clusters **Score 4**= several primary and secondary oocytes mixed with testicular tissue; presence of ovarian cavity and the absence of spermatic duct **Score 5**= extended areas of testicular tissue intermingled with female gonadal tissue <50%, with primary and/or secondary oocytes; presence of single ovarian cavity **Score 6**= female gonadal tissue >50% with primary and/or secondary oocytes; presence of single ovarian cavity **Score 7**= normal ovary	*Rutilus rutilus*
Jobling *et al.,* 2006	**Score 0**= normal testis **Score 1**= multifocal ovotestis with 1-5 oocytes (single) **Score 2**= multifocal ovotestis with 6-20 oocytes (clusters) **Score 3**= multifocal ovotestis with 21-50 oocytes (clusters) **Score 4**= multifocal oocytes 50-100 (clusters) **Score 5**= focal or multifocal aggregate of oocytes > 100 **Score 6**= more than 50% is ovarian tissue **Score 7**= 100% is ovarian tissue	*Rutilus rutilus*
Blazer *et al.*, 2007	**Score 1**= 1 oocyte in 1 field **Score 2**= ≥1 oocyte in 1 field **Score 3**= 2-5 oocytes in clusters **Score 4**= ≥5 oocytes in clusters	*Micropterus*

As reported by Hinck *et al.* (2009), that investigate the prevalence of intersex in 16 species of fresh water fish from rivers located in 9 different regions of the US, only 4 fish species showed intersex (catfish, common carp, largemouth bass and perch). This different susceptibility may be due to different mechanisms of sexual differentiation in fish species. In particular, gonadal differentiation is influenced by both genetic and environmental factors (i.e temperature and water pH). It is also important to consider that variables such as season and age of fish sampled may be responsible for the differences observed in the prevalence of intersex between species. In fact, a seasonal pattern was evidenced in wild *L. ramada* by Tancioni *et al.* (2015) with high values of intersex recorded during the spawning and gonad development periods. Moreover the timing of exposure to pollutants can be critical as fish seem to be most susceptible to EDCs just after hatching or as juveniles before sex differentiation (Jobling *et al.*, 1998; Bateman *et al.*, 2004).

Estrogenic effects caused by EDCs were also observed in the swordfish (*Xiphias gladius*) (De Metrio *et al.*, 2003), in bluefin tuna (*Thunnus thynnus*), little tunny (*Euthynnus alletteratus*) and red mullet (*Mullus barbatus*) from the Mediterranean Sea (Fossi *et al.*, 2002; Martin-Skilton *et al.,* 2006; Macías *et al.*, 2014).

Similarly in Germany, Belgium, Poland, France and Spain cases of the intersex in freshwater fish have increased substantially in recent years. Precisely, studies conducted in Germany revealed the presence of intersex in the common bream (*Abramis brama*), in the perch (*Perca fluviatilis*) and the three-spined stickleback (*Gasterosteus aculeatus*). In Belgium, this condition was instead recognized in the stone loach (*Barbatula barbatula*) while in Poland in the grayling (*Thymallus thymallus*), belonging to the family Salmonidae. Furthermore in France intersex cases were observed in the European chub (*Squalius cephalus*) while in Spain gonadal abnormalities have been reported in common carp (*Cyprinus carpio*). Both of these countries have recently conducted research on marine populations of Mugilidae family, detecting the presence of intersex in *Chelon labrosus* in polluted estuaries of the Basque Coast (Puy-Azurmendi *et al.*, 2013).

About Italy, some studies have shown the presence of intersex in the Italian barbel (*Barbus plebejus*) sampled in the Po river (Viganò *et al.*, 2001; 2006). However, recently, Tancioni *et al.* (2015) conducted several studies on specimens of the *Liza ramada* species, highlight the presence of intersex in mugilids living in water contaminated by urban discharges of the river Tiber, in the Tyrrhenian side of central

Italy.

Generally hermaphroditism and intersex are not frequently found conditions in striped mullets that live in uncontaminated water environments (McDonough *et al.*, 2005). However to date, numerous studies have shown the presence of gonadal disorders in many species of Mugilidae, attributing this event to EDCs exposure (Puy-Azurmendi *et al.*, 2013; Bizarro *et al.*, 2014). In other instances, in the Adriatic region of Italy where contaminated water is a serious issue, skeletal anomalies in Mugilids species were observed (Boglione *et al.*, 2006). Accordingly Mugilidae can be considered as sentinel species in coastal biomonitor investigations (Waltham *et al.*, 2013) and in particular of exposure to EDCs in coastal and estuarine polluted environments (Ortiz-Zarragoitia *et al.*, 2014 and references therein).

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Chapter 2

SAMPLING SITES AND MULLET SPECIES. PROCESSING TECHNIQUES AND MORPHOMETRIC FEATURES OF THE FISH EXAMINED

2.1 Study area

Sardinia Island (central-western Mediterranean Sea, Italy) is characterized by approximately 80 wetlands covering a surface of about 15.000 hectares. Extensive aquaculture is a typical activity in many of these biotopes, mainly for euryhaline fish like mullets (Cannas *et al.,* 1998). In this study, four brackish habitats were examined: the Cabras and the Marceddì lagoons (Central western Sardinia, Fig. 2.1) the Calich lagoon (North western Sardinia, Fig. 2.1) and the San Teodoro lagoon (North eastern Sardinia, Fig. 2.1).

Fig. 2.1 Study area and sampling sites.

The above mentioned brackish environments will be described in detail in view of the geography of the territory and the distribution of production sites.

2.2 Lagoon characteristics

2.2.1 North western Sardinia

It is the part of Sardinia less rich in ponds and coastal lagoons, since the different wetlands have reduced dimensions and limited significance from the point of view of fish production. The interest is more naturalistic and ornithological, since nesting and wintering habitat of migratory birds. In the peninsula of Cape Falcone there are three

small ponds: Saline, Casaraccio and Pilo. In the municipality of Alghero there is the Calich lagoon.

2.2.2 The Calich lagoon

The Calich lagoon (40°36′N 8°18′E) is located along the northwest coast of Sardinia behind the town of Alghero. It is a coastal wet system of 92 hectares for a length of 2.650 meters (Fig. 2.2).

Fig. 2.2 The Calich lagoon (photo from Fenza *et al.*, 2014).

The Calich lagoon extends from the periphery of Alghero to the village of Fertilia. It communicates with the sea through a large canal (port of Fertilia) sixty meters wide and two meters deep and where the remains of a Roman bridge are present. Three waterways feed the lagoon: the Oruni channel 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 (Fenza *et al.,* 2014). The limited supply of fresh water has favoured an increase in salinity of the lagoon with the resulting adaptation of flora and terrestrial and marine wildlife. Different selected point of the lagoon were sampled to measure salinity, temperature, pH, nitrites/nitrates, phytoplankton components and heavy metal concentrations because in 2010 numerous episodes of anomalous discoloration of waters were observed in the Calich lagoon. Collected data are in agreement with an increase in nutrients (nitrogen and phosphorus) that could be related to the dramatic microalgae growth detected. For this reason, the best use of depuration plants of wastewaters and agricultural wastes should be

considered to preserve this ecosystem (ARPAS, 2014). Although the area of the Calich lagoon is less extended and fish productive rates are lower compared to other wetlands of Sardinia, seafood production is quite varied and mullet, sea bream, sea bass, sole, eel, crabs are fished with a yield estimated at approximately 200 kg/ha/year. In the lagoon a modern "lavoriero" was built but the cooperative does not used it and fishermen prefer to fish with gillnets. The lagoon system was given in concession to the "Cooperativa Pescatori Algheresi il Golfo e la Laguna" and it is considered an oasis of wildlife protection and capture, belonging to the Natural Regional Park of Porto Conte (Fenza *et al.,* 2014).

2.2.3 North eastern Sardinia

In this part of the Sardinian coast going from Santa Teresa to Orosei are present numerous wetland of natural beauty, which represent an ideal habitat for migratory birds. The fish production sites of major interest are the Porto Pozzo, the San Teodoro lagoon, the Sa Curcurica lagoon, the compendiun of Cedrino and Avalè-Su Petrosu.

2.2.4 The San Teodoro lagoon

The San Teodoro lagoon (40°47′51.71″N 9°40′00.05″E) covers a surface area of 218 hectares and is located near the municipality of San Teodoro (Fig. 2.3).

This lagoon is 3.5 km long and with a depth average of 0.7 meters. Punta Sabbatino borders the lagoon in the north and the cordon dune of the Cinta beach in the east. Several tributaries feed this lagoon the main of which is the Rio San Teodoro. This lagoon is composed of two basins, the proper lagoon (200 ha) and the Pescaia basins (30 ha) that connect the lagoon to the sea. Sandy or muddy beds with granitic rock formation visible on the water surface characterize the lagoon.

The San Teodoro lagoon is located in two areas relevant for biodiversity (Gallura and Baronia), well known for richness in numerous vegetal and animal species. For these reasons, the San Teodoro lagoon is considered a wetland of international importance and a place where to protect species at risk of extinction. However, the small town of San Teodoro (one of the most important tourist centre in the North-eastern coast of the Island) frequently discharges into it untreated wastewaters that potentially could affect the lagoon creating a significant impact on the environment.

These factors represent the major cause of eutrophication of waters in this lagoon (Munari & Mistri, 2007). Although this lagoon is still considered scarcely exploited for

fishing several fished species (mullets, eels, sea basses, sea breams and flounders) are captured trough the use of lavorieri. In addition there is a shellfish production of clams and oysters. The owner of the lagoons is the municipality of San Teodoro that released fish license to a local cooperative (Fenza *et al.,* 2014).

Fig. 2.3 The San Teodoro lagoon.

2.2.5 Central western Sardinia

In the central part of the west coast is located the area of Oristano, one of the most rich in wetlands, with a total area of about 6.000 hectares of water surface. Around the Gulf of Oristano there are among the most productive ponds and lagoons of the island: San Giovanni - Marceddì, Pauli Biancu Turri, Corru S'Ittiri, S'Ena Arrubia, Santa Giusta, Cabras , Mistras, Is Benas.

2.2.6 The Cabras lagoon

The Cabras lagoon is located in the Gulf of Oristano (39°57′00″N 8°29′06″E) and is the largest lagoon system in Sardinia. Its surface area is approximately 2.230 hectares, and it is located in the municipality of Cabras, Nurachi and Riola Sardo (Fig. 2.4).

The bottom of the lagoon is predominantly muddy and is characterized by an average depth of around 1.6 meters and a maximum depth of around 3 meters (Lugliè *et al.,* 2012). The lagoon communicates with the sea through four small channels and receives fresh water from two rivers: the Rio 'e Mare Foghe and the Rio Tanui. In the 80's human intervention made drainage canal to prevent, in the event of heavy rainfall, flooding in the town of Cabras, changing progressively the lagoon environment (Fenza

et al., 2014). Moreover, this ecosystem suffered by an intense anthropic pressure due to agricultural activities and municipal wastewaters altering the status of eutrophication in the lagoon. In the Cabras lagoon, extensive aquaculture is an important economical resource that in 1998 has reached a fish production around 850 tons that has fallen to 80 tons in 1999. This severe decrease of production was caused by a eutrophication process that changed salinity and water temperature with an irregular trend in the following years (Lugliè *et al.,* 2012). Capture techniques are both traditional (i.e a traditional weir made by wooden posts and reeds in the Mare 'e Pontis) and modern (i.e by the use of lavorieri) (Fenza *et al.,* 2014). In the Cabras lagoon, the most captured fish species are mullets, eels, sea breams and sea basses. The lagoon was given in concession to the "Concessionario - Nuovo Consorzio Cooperative Pontis" and it is considered an oasis of wildlife protection and capture, under the Ramsar Convention Secretariat (2013) (Fenza *et al.,* 2014).

Fig. 2.4 The Cabras lagoon (photo from Fenza *et al.*, 2014).

2.2.7 The Marceddì-San Giovanni lagoon

Corru s'Ittiri, San Giovanni and Marceddì are part of the named "Corru s'Ittiri" pond. The lagoon system is located in the southern part of the Gulf of Oristano (39°42′40.01″N 8°31′06.53″E) near Arborea Terralba, Guspini and Arbus (Fig. 2.5).

The Corru s'Ittiri lagoon covers a surface area of 120 hectares and its depth vary from 40 cm up to 2 meters. It has no natural tributaries, but receives the waters that come from the agricultural land of the plain of Arborea, through irrigation canals. It is separated from the sea by a barrier, in part natural, where "lavorieri" have been built.

The compendium of San Giovanni and Marceddì can be considered as a single large cove, with a total area of 1.600 hectares and depth varying from 40 cm up to 2 meters divided by means of an artificial barrier. San Giovanni (about 700 hectares) is located in the inner part of the compendium and is characterized by more fresh water (Rio Mogoro, Rio Mannu and Rio Sitzerri), while Marceddì (about 900 hectares) located in the outer part has an extensive communication with the sea. Important hydraulic works operated in the 90's and the construction of a driveway barrier, near the village of Marceddì fishermen, caused a reduction of water exchange with the sea, thus resulting in an overall lowering of the salinity of the lagoon system.

The Marceddì lagoon is located in an area of intensive agricultural and zootechnical activities and receives wastewaters from the surrounding watershed which can be a potential source of contaminants. The prevalent fish species are sea bream, sea bass, mullets and eels. Crab, mussels and clams are also an important economical resource. The lagoon system was given in concession to the "Consorzio Cooperative Riunite della Pesca di Marceddì" that operates throughout the lagoon system Corru s'Ittiri, San Giovanni and Marceddì. This system, already identified under the Ramsar Convention Secretariat (2013) as "wetlands of international importance" is an oasis of wildlife protection and capture (Fenza *et al.,* 2014).

Fig. 2.5 The Marceddì lagoon.

2.3 Mugilidae species of interest

2.3.1 *Chelon labrosus*

Chelon labrosus (Risso, 1827), (Order Perciformes, family Mugilidae, genus *Chelon*)

commonly called thicklip grey mullet. Its economic importance varies in Mediterranean countries (Fig. 2.6)

General characteristic: *C. labrosus* has a slim and fusiform body with almost circular section. Upper lip is a distinctive feature because it is broad with 1-4 rows of papille. Adipose eyelid is very small and jugular space is very narrow. Dorsal spines are 5 and anal 3. The pyloric caeca are 6. Length is generally around 32 cm but female can reach 75 cm in length (Hayward & Ryland, 1990; www.fishbase.se/summary/Chelon-labrosus.html). Biology: adults move in schools and frequently entering in brackish lagoons and freshwater. They migrate in summer as temperatures rise. They are able to tolerate huge changes of water salinity, and they are found both on muddy and sandy bottom (Fenza *et al.,* 2014) Reproduction occurs in the sea between January and March. Distribution: *Chelon labrosus* is distributed along the Mediterranean Sea and South-western Black Sea, North-eastern Atlantic Coasts, including the British Isles, North Sea, Norwegian Sea, Barents Sea, Baltic Sea, Bay of Biscay, in the Canaries, Azores and Madeira, and coasts of West Africa (Crosetti & Blaber, 2016 and the references therein). Food and feeding: adults migrate continuously to the lagoon in search of food (Arechavala-Lopez *et al.,* 2010). They feed on benthic diatoms, algae, small molluscs, crustaceans and detritus. Fry and juveniles only feed on zooplankton and this activity is mainly concentrate in the morning (Tosi & Torricelli, 1988).

Fig. 2.6 *Chelon labrosus*.

2.3.2 *Liza aurata*

Liza aurata (Risso, 1810), (Order Perciformes, family Mugilidae, genus *Liza*) commonly known as golden grey mullet. Among the species of the genus is the one that has the greatest commercial value (Fig. 2.7).

General characteristic: the name "*aurata*" indicate the characteristic well defined golden spot present on the operculum. Body is fusiform, the head is a little depressed and the upper lip is thin and smooth. The adipose eyelid is very small and the jugular space is

ovoid in shape. The pectoral fin reaches the orbit when turned forward and no evident dark spot is present at the pectoral axil. Dorsal spines are 5 and anal 3. The pyloric caeca are 8 and the dorsal one is the longest. Length is generally around 30 cm where females can reach a maximum of 34 cm and males 59 cm (Hayward & Ryland, 1990; fishbase.org/summary/Liza-aurata.html).

Biology: *Liza aurata* is the species among Mugilidae least tolerant of freshwater. Adults usually live in schools, entering lagoons and lower estuaries. Juveniles move in this area in winter and especially in spring. *Liza aurata* is a catadromous species that spawns in late summer-november in the sea. As other Mugilidae species female produce eggs in a range from 80.000 to 1.400.000 (Hotos *et al.,* 2000; Fenza *et al.,* 2014). Distribution: *Liza aurata* is found in the Mediterranean and the Black Sea, North-eastern Atlantic coasts, including the British Isles, North Sea, Norwegian Sea, Barents Sea, Bay of Biscay, the offshore islands of the Canaries, Azores and Madeira, and coasts of West Africa and its presence was reported also in the Caspian Sea (Crosetti & Blaber, 2016 and the references therein). Food and feeding: adults feed on small benthic organisms, detritus, and occasionally on insects and plankton. Fry feed exclusively on zooplankton mainly in the morning (Tosi & Torricelli, 1988).

Fig. 2.7 *Liza aurata*.

2.3.3 *Liza ramada*

Liza ramada (Risso, 1827) (Order Perciformes, family Mugilidae, genus *Liza*) is also known as thinlip grey mullet (Fig. 2.8).

General characteristic: the body is fusiform, with a robust head and flattened profile. Upper lip is thin, adipose eyelid is not present and the jugular space is ovoid in shape. The pectoral fins not reached the orbit if turned towards the head. An indistinct golden spot on operculum and a black spot at the pectoral axil characterized this specie. Dorsal fins are two and well separated with 4-5 spines while anal spines are generally 3. Seven uniform in length pyloric caeca are present. Length is generally around 35 cm, even if

specimens of 70 cm have been observed (Hayward & Ryland, 1990; www.fishbase.se/summary/Liza-ramada.html). Biology: adults are pelagic and are found along the coast and in ponds and lagoons. They prefer temperatures of 8-24°C and lower level of salinity so that they can live also into freshwater They move in schools to the lagoons often in polluted waters, while juveniles inhabit the coast and estuaries. Spawning occurs from September to February and adults undergo migration to the sea (Fenza *et al.,* 2014). Distribution: *L. ramada* is largely diffuse in the Mediterranean and the Black Sea, North-eastern Atlantic coasts, including the British Isles, North Sea, Norwegian Sea, Barents Sea, Bay of Biscay, the offshore islands of the Canaries, Azores and Madeira, and coasts of West Africa down to Mauritania (Crosetti & Blaber, 2016 and references therein) Food and feeding: adults of *Liza ramada* are pelagic and feed on micro algae, small benthic or planktonic organisms as well as organic detritus (Almeida, 1996).

Fig. 2.8 *Liza ramada.*

2.3.4 *Mugil cephalus*

Mugil cephalus (Linneo, 1758) (Order Perciformes, family Mugilidae, genus *Mugil*), also known as grey mullet, striped mullet or sea mullet, represents the most appreciated species among Mugilidae family (Fig. 2.9). General characteristic: the body is firm, cylindrical in cross-section and slightly compressed on the sides. The head is enlarged and obtuse, lips are smooth and thin. A distinctive character from the other mullets is the eye covered by thick adipose membrane that covers most of the pupil (Fenza *et al.,* 2014). The pectoral fins are rounded and a dark spot is visible. Dorsal fins are in number of 5 and anal spines are 3. *M. cephalus* varies from the other mullet species by pyloric caeca that are 2 in number. Length is generally lower than 50 cm but they can reach also 100 cm in length (Hayward & Ryland, 1990; www.fishbase.org/summary/MUGIL-CEPHALUS.html). Biology: adults are frequently found in large shoals in estuarine and marine coastal areas. They stay on sand or mud

bottom in tropical, subtropical and temperate waters. Spawning season goes from August to October and females spawn 0.8 to 2.6 million eggs at sea. At 3 to 4 years they are sexually mature. In some place, *Mugil cephalus* is cultured especially for the production of mullet roe, an expensive delicatessen (bottarga), although their fillet is appreciated because rich in protein and vitamins (Whitfield *et al.,* 2012; Fenza *et al.,* 2014). Disribution: *Mugil cephalus* is the most common and cosmopolitan member of the Mugilidae family (Whitfield *et al.,* 2012; Waltham *et al.,* 2013) and are found along the Mediterranean and Black Sea coasts, North-eastern Atlantic Coasts, including the British Isles Coasts, North Sea, Norwegian Sea, Barents Sea, Bay of Biscay, the offshore islands of the Canaries, Azores and Madeira, and coasts of West Africa (Crosetti & Blaber, 2016 and references therein). Food and feeding: adult *M. cephalus* privileged muddy or sandy bottoms as they mainly feeds on detritus, micro algae and benthic organisms. Juveniles are generally zooplanktonic and became detritivorous when increase in size. Both adults and fry of *M. cephalus* feed during morning or sunset on benthonic organisms (Tosi & Torricelli, 1988).

Fig. 2.9 *Mugil cephalus*.

2.4 Sampling techniques

Four hundred and ninety-five adult specimens belonging to the family Mugilidae (*Chelon labrosus*, *Liza aurata*, *Liza ramada* and *Mugil cephalus*) were sampled during summer and autumn of 2013, 2014 and 2015. However, during the first year of sampling, mullets were collected only in two lagoons (Cabras and San Teodoro), while in autunn 2015 mullets were fished only in the Calich lagoon. All the mullet species were sampled in all lagoons except for Cabras, where only *L. ramada* was collected.

Mugilidae were sampled before 11.00 a.m. in the capture chambers of fixed traps (Cataudella *et al.*, 2015). Fish were immediately euthanatized by overexposure to Tricaine Methanesulfonate (MS-222), kept on tanks with ice and transported to the laboratory within 2 hours after capture. All the specimens were classified at species

level following Farrugio (1977), weighed for total weight, measured for total length and photographed.

2.5 Necropsy

Gross examination of each subject was performed to evaluate the presence of macroscopic lesions on the body surface. Any abnormalities including areas with discoloration, increased mucus, skin ulceration, haemorrhages were also evaluated (Fig. 2.10).

Fig. 2.10 *Mugil cephalus.* Operculum: skin ulceration.

For necropsy each fish was disposed in right lateral side and the first incision was made with a sterile scissors on the mid-line cranially to the pelvic girdle being careful to avoid faecal contamination from intestine rupture. Then the cut was continued from the abdominal wall to the mandibular branch. The second incision was made from the pelvic girdle moving dorsally and then cranially to reach the operculum. At this point the abdominal wall and the operculum were removed showing all the visceral organs (Fig. 2.11).

Some organs (liver and gonads) were weighted and heart, liver, spleen, gonads, kidney, air bladder, pyloric caecae and entire gastro intestinal tract were macroscopically examined for abnormalities such as discoloration (i.e. lipidosis), enlargement (hypertrophy), abnormalities, haemorrhage, abscesses and nodules (Fig. 2.12).

Fig. 2.11 *Liza ramada*. Necropsy of female mullet.

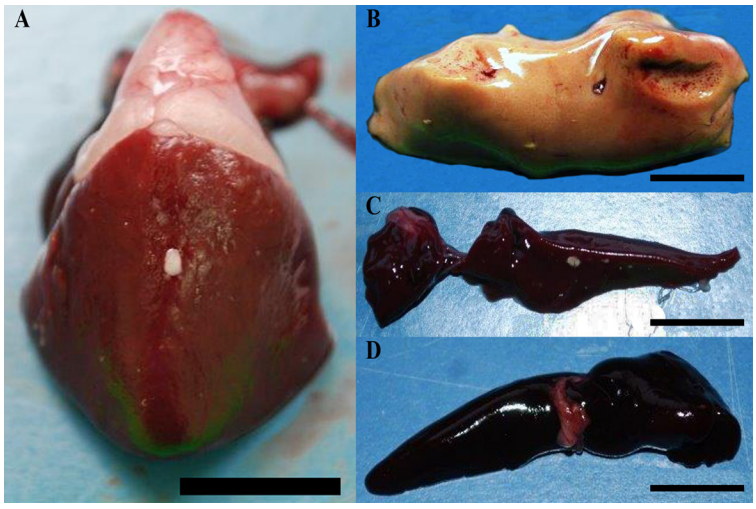


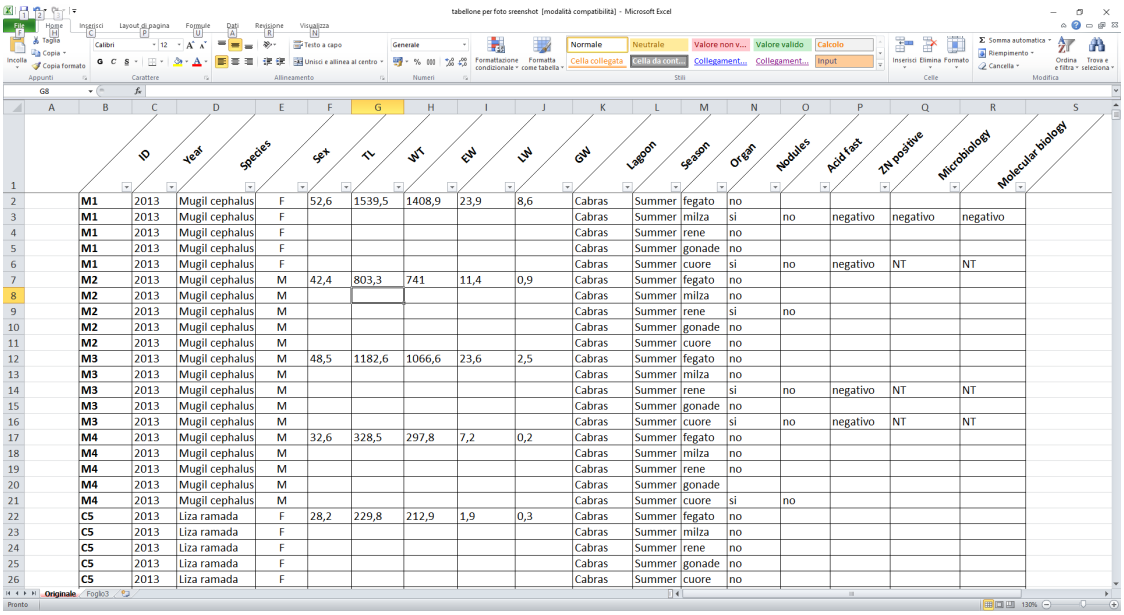
Fig. 2.12 Gross examination of visceral organs. (A) Mullet heart: withe small nodule round in shape within myocardium. (B) Mullet liver: lipidosis and two small nodules on the organ sufarce. (C) Mullet spleen: focal roundish white nodule within parenchima. (D) Mullet spleen: enlargement of the organ (splenomegaly). Bar=1 cm.

2.6 Histopathology

Samples of target organs (liver, spleen, heart and kidney) and portions from anterior,

middle and posterior part of both right and left gonads were collected from each specimen for subsequent histopathological examination. In addition, portions of liver and spleen were placed into Petri dishes and stored at -20°C for bacteria identification by specific analysis, as described in detail in Chapter 3 (Material and methods section). Extra samples of spleen were also collected in 1.5 ml tube and stored at -80°C for DNA extraction and specific PCRs, as described in detail in Chapter 3 (Material and methods section).

Tissues from each mullet were identified with a progressive number and an Excel database including data of each specimen as fish identification number (ID), years, species, sex, total length (TL), total weight (TW), eviscerated weight (EW), liver weight (LW), gonad weight (GW), lagoon, season, organ, nodule, acid fast bacilli, positivity to Ziehl-Neelsen stain (ZN), microbiology and molecular biology was created (Fig. 2.13).



ID	Year	Species	Sex	TL	WT	EW	LW	GW	Lagoon	Season	Organ	Nodules	Acid fast	ZN positive	Microbiology	Molecular biology
M1	2013	Mugil cephalus	F	52,6	1539,5	1408,9	23,9	8,6	Cabras	Summer	fegato	no				
M1	2013	Mugil cephalus	F						Cabras	Summer	milza	si	no	negativo	negativo	negativo
M1	2013	Mugil cephalus	F						Cabras	Summer	rene	no				
M1	2013	Mugil cephalus	F						Cabras	Summer	gonade	no				
M1	2013	Mugil cephalus	F						Cabras	Summer	cuore	si	no	negativo	NT	NT
M2	2013	Mugil cephalus	M	42,4	803,3	741	11,4	0,9	Cabras	Summer	fegato	no				
M2	2013	Mugil cephalus	M						Cabras	Summer	milza	no				
M2	2013	Mugil cephalus	M						Cabras	Summer	rene	si	no			
M2	2013	Mugil cephalus	M						Cabras	Summer	gonade	no				
M2	2013	Mugil cephalus	M						Cabras	Summer	cuore	no				
M3	2013	Mugil cephalus	M	48,5	1182,6	1066,6	23,6	2,5	Cabras	Summer	fegato	no				
M3	2013	Mugil cephalus	M						Cabras	Summer	milza	no				
M3	2013	Mugil cephalus	M						Cabras	Summer	rene	si	no	negativo	NT	NT
M3	2013	Mugil cephalus	M						Cabras	Summer	gonade	no				
M3	2013	Mugil cephalus	M						Cabras	Summer	cuore	si	no	negativo	NT	NT
M4	2013	Mugil cephalus	M	32,6	328,5	297,8	7,2	0,2	Cabras	Summer	fegato	no				
M4	2013	Mugil cephalus	M						Cabras	Summer	milza	no				
M4	2013	Mugil cephalus	M						Cabras	Summer	rene	no				
M4	2013	Mugil cephalus	M						Cabras	Summer	gonade					
M4	2013	Mugil cephalus	M						Cabras	Summer	cuore	si	no			
C5	2013	Liza ramada	F	28,2	229,8	212,9	1,9	0,3	Cabras	Summer	fegato	no				
C5	2013	Liza ramada	F						Cabras	Summer	milza	no				
C5	2013	Liza ramada	F						Cabras	Summer	rene	no				
C5	2013	Liza ramada	F						Cabras	Summer	gonade	no				
C5	2013	Liza ramada	F						Cabras	Summer	cuore	no				

Fig. 2.13 Database containing all the relevant data collected.

Samples were placed in labelled cassettes and fixed in 10% neutral formalin for 48h. Tissue sections were dehydrate with increasing concentrations of alcohol and xylene in an automatic tissue processor (HISTO-PRO 200) and paraffin embedded (Fig. 2.14)

Fig. 2.14 Automatic tissue processor.

The protocol proposed by Mazzi (1977) was used according to the histological procedures:

- Fixation: samples were treat in a liquid fixing agent. Formaldehyde commercially available in solution of 40% was buffered with CaCO3 and 10% neutral formalin was used to fix the tissues. This fixative penetrate tissue causing chemical and physical changes increasing their hardness. Specimens were placed in labelled cassettes (small perforated baskets) to separate them from other specimens. The duration of the processing schedule was 48 h in order to prevent the autolysis and degradation of the tissue.
- Dehydration: samples were immerse in a series of ethanol solutions of increasing concentration to remove the water and to allow melted paraffin infiltrate the tissue:
  1. 50% ethanol (2h)
  2. 70% ethanol (2h)
  3. 90% ethanol (2h)
  4. 100% ethanol (2h)
  5. 100% ethanol (2h)
- Clearing: this processes provide the use of xylene, an intermediate solvent that is miscible with both ethanol and paraffin wax. This solvent has an important role to remove also a substantial amount of fat from the tissue. When ethanol has been entirely replaced by xylene, the tissue has a translucent appearance, so this

is called clearing agent. Two clearing sequence were made:

1. xylene (2h)
2. xylene (2h)

- Wax infiltration: in this phase tissue cassettes were dipped in 3 steps of paraffin, a mixture of hydrocarbons with a melting point at 60°C. At the end of this process the paraffin entirely infiltrated the tissue and replaced xylene. Three wax infiltration were made:
  1. Wax infiltration (2h)
  2. Wax infiltration (2h)
  3. Wax infiltration (2h)
- Embedding or blocking out: the tissue is embedded within a block of paraffin, using an embedding centre (ACM 50; Fig. 2.15), where a mould is filled with molten wax and the specimen placed into it (Fig. 2.16). It is very important to orientate the specimens to obtain a well oriented plane of section. (Fig. 2.17).

Fig. 2.15 Embedding centre (ACM 50).

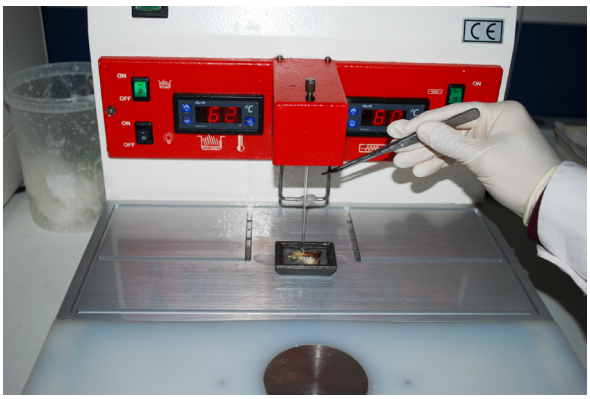


Fig. 2.16 Tissue into a mould filled with molten paraffin.

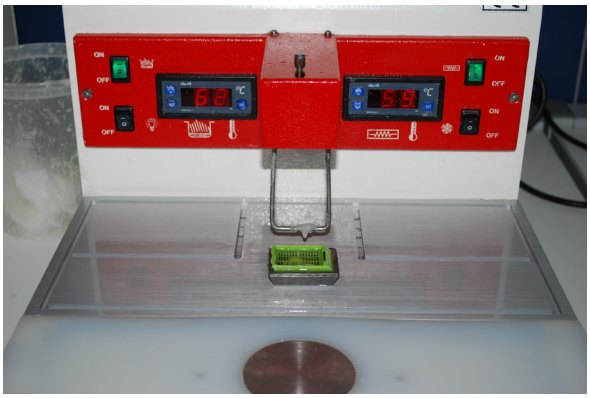


Fig. 2.17 Tissue sample well oriented.

- Cut and stained: the paraffin solidified blocks were removed from the mould and were cut with the microtome (Leica RM 2245) (Fig. 2.18). Sections of 3µm were obtained and were stained in an automatic multistainer (ST5020, Leica Biosystems) (Fig. 2.19) with Hematoxylin and Eosin (HE) according to a standard method (Mazzi 1977). Sections were also stained with Ziehl-Neelsen stain (ZN) to detect acid fast bacilli (Bancroft & Gamble, 2002). Sections were mounted on a glass slide and then evaluated at light microscopy (Nikon Eclipse 80i) (Fig. 2.20).

Fig. 2.18 Microtome and paraffin blocks.

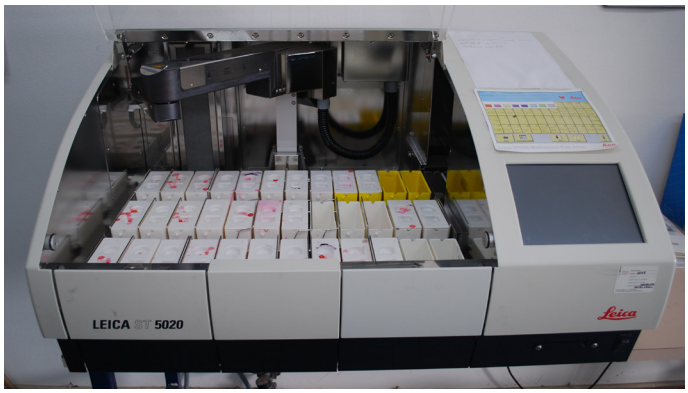


Fig. 2.19 Automatic multistainer.

Fig. 2.20 Evaluation of specimens at light microscope.

The histological protocols used for staining sections are reported below:

Haematoxylin and eosin (H&E)

1. Slides were deparaffinized and rehydrated through graded alcohols
2. Harris' hematoxylin (3 min)
3. Washed in running tap water (5 min x 2)
4. Rinsed in distilled water
5. Eosin (2 min)
6. Rinsed in distilled water
7. Dehydrated, cleared and mounted

Ziehl-Neelsen stain (ZN)

1. Slides were deparaffinized and rehydrated through graded alcohols
2. Carbol-fuchsin (15 minutes at 58°C)
3. Washed in tap water
4. Rinsed in distilled water
5. Differentiated in 1% acid alcohol (30 seconds)
6. Washed in tap water and then in distilled water
7. Counterstained in methylene blue solution (30 seconds)
8. Differentiated in ethanol until the background was pale blue
9. Dehydrated cleared and mounted

2.7 Results

Four hundred and ninety-five adult specimens belonging to the family Mugilidae [*Chelon labrosus* (54 fish), *Liza aurata* (141 fish), *Liza ramada* (248 fish) and *Mugil cephalus* (52 fish)] were sampled twice a year (summer and autumn) from July 2013 to October 2015. All the mullet species were sampled at Calich (120 fish), Marceddì (89 fish) and San Teodoro (138 fish) lagoons. At Cabras, instead, only *L. ramada* and *M. cephalus* (148 fish) were caught (Table 2.1). In detail, Table 2.2 illustrates the number of mullet specimens sampled from the 4 lagoons during summer and autumn of the years 2013, 2014 and 2015, respectively.

Table 2.1 Specific abundance of the mullet species sampled at the 4 Sardinian lagoons.

	C. labrosus	*L. aurata*	*L. ramada*	*M. cephalus*	Total
Cabras	-	-	144	4	148
Calich	20	5	93	2	120
Marceddì	8	74	2	5	89
San Teodoro	26	62	9	41	138
Total	54	141	248	52	495

Table 2.2 Yearly and seasonally abundance of the mullet species sampled at the 4 Sardinian lagoons.

Year	Season	Species	Sampling site				
			Cabras	Calich	Marceddì	San Teodoro	Total
2013	Summer	*Chelon labrosus*	-	-	-	8	8
		Liza aurata	-	-	-	20	20
		Liza ramada	26	-	-	-	26
		Mugil cephalus	4	-	-	2	6
			30	-	-	30	60
	Autumn	*Chelon labrosus*	-	-	-	6	6
		Liza aurata	-	-	-	-	-
		Liza ramada	28	-	-	5	33
		Mugil cephalus	-	-	-	7	7
			28	-	-	18	46
Total of the year			58	-	-	48	106
2014	Summer	*Chelon labrosus*	-	16	-	10	26
		Liza aurata	-	1	29	13	43
		Liza ramada	30	13	-	1	44
		Mugil cephalus	-	-	-	6	6
			30	30	29	30	119
	Autumn	*Chelon labrosus*	-	-	8	2	10
		Liza aurata	-	-	17	23	40
		Liza ramada	30	30	2	1	63
		Mugil cephalus	-	-	3	4	7
			30	30	30	30	120
Total of the year			60	60	59	60	239
2015	Summer	*Chelon labrosus*	-	1	-	-	1
		Liza aurata	-	3	28	6	37
		Liza ramada	30	24	-	2	56
		Mugil cephalus	-	2	2	22	26
			30	30	30	30	120
	Autumn	*Chelon labrosus*	-	3	-	-	3
		Liza aurata	-	1	-	-	1
		Liza ramada	-	26	-	-	26
		Mugil cephalus	-	-	-	-	-
			-	30	-	-	30
Total of the year			30	60	30	30	150

2.7.1 Cabras

Overall, 148 mullets were examined. *Liza ramada*: 144 specimens (TL=32.7±2.6 cm; TW=310.7±76.4 g), of which 65 were females (TL=32.7±2.8 cm; TW=310.9±90.5 g; Figs. 2.21 & 2.22) and 79 were males (TL=32.7±2.4 cm; TW=310.5±63.1 g; Figs. 2.23 & 2.24).

Mugil cephalus: 4 specimens, of which only 1 was a female (TL=52.6 cm; TW=1539.5 g) and 3 were males (TL=41.2±8.0 cm; TW=771.5±427.9 g).

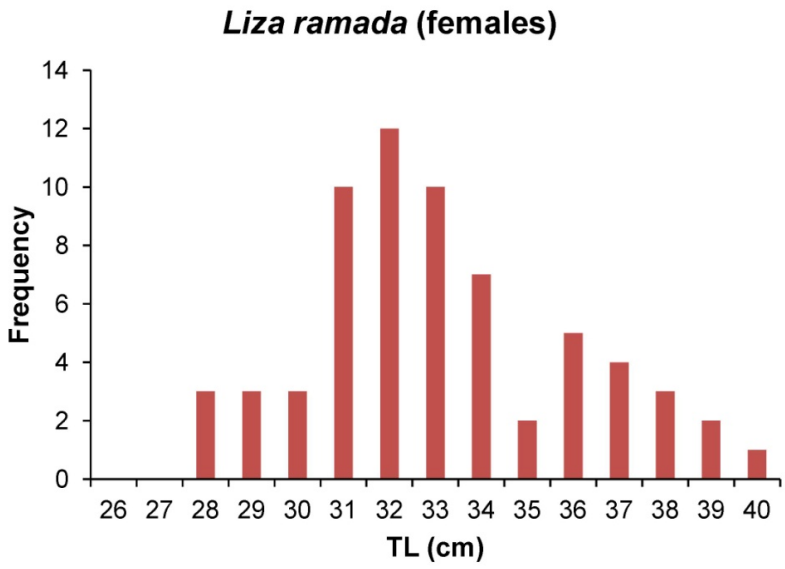


Fig. 2.21 Distribution of length size classes of *Liza ramada* at the Cabras lagoon.

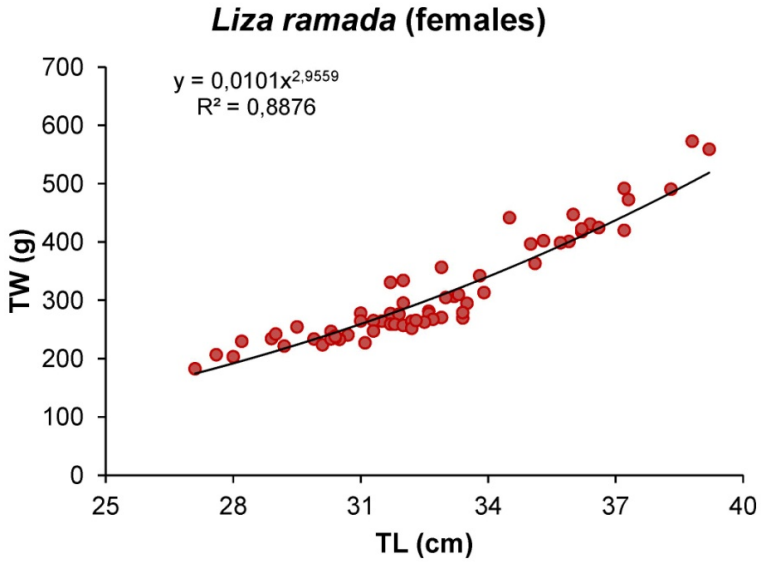


Fig. 2.22 Length-weight relationship of *Liza ramada* at the Cabras lagoon.

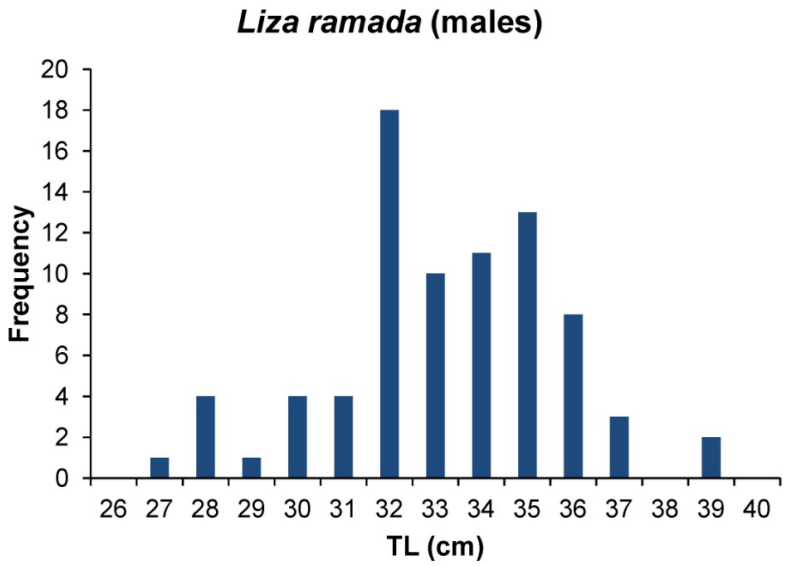


Fig. 2.23 Distribution of length size classes of *Liza ramada* at the Cabras lagoon.

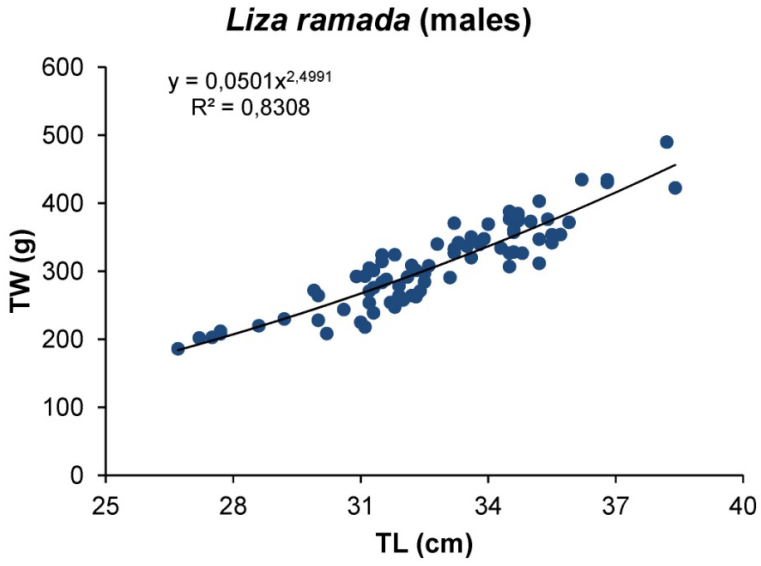


Fig. 2.24 Length-weight relationship of *Liza ramada* at the Cabras lagoon.

2.7.2 Calich

On the whole, 120 mullets were examined. *Chelon labrosus*: 20 specimens (TL=33.7±3.3 cm; TW=412.3±160.6 g), of which 15 were females (TL=33.8±3.4 cm; TW=419.8±172.0 g; Figs. 2.25 & 2.26), 4 were males (TL=33.8±3.8 cm; TW=417.0±139.0 g) and 1 was classified as intersex (TL=30.9 cm; TW=282.1 g).

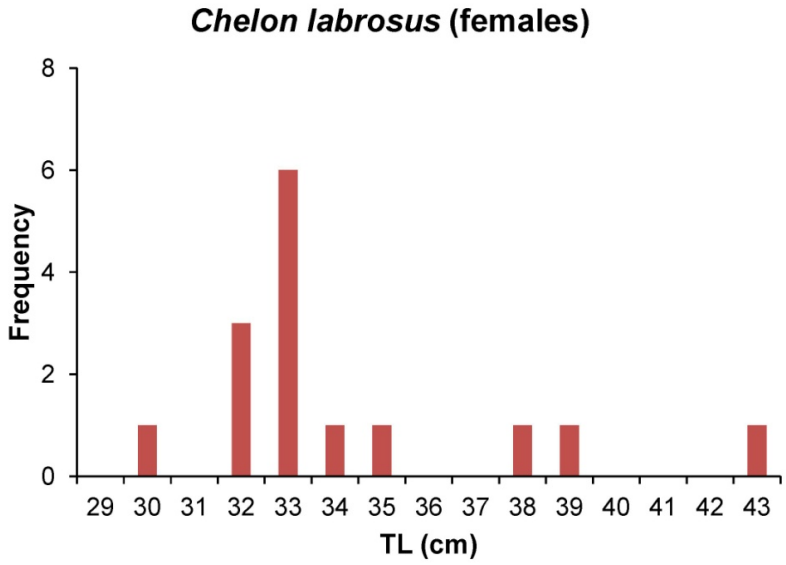


Fig. 2.25 Distribution of length size classes of *Chelon labrosus* at Calich lagoon.

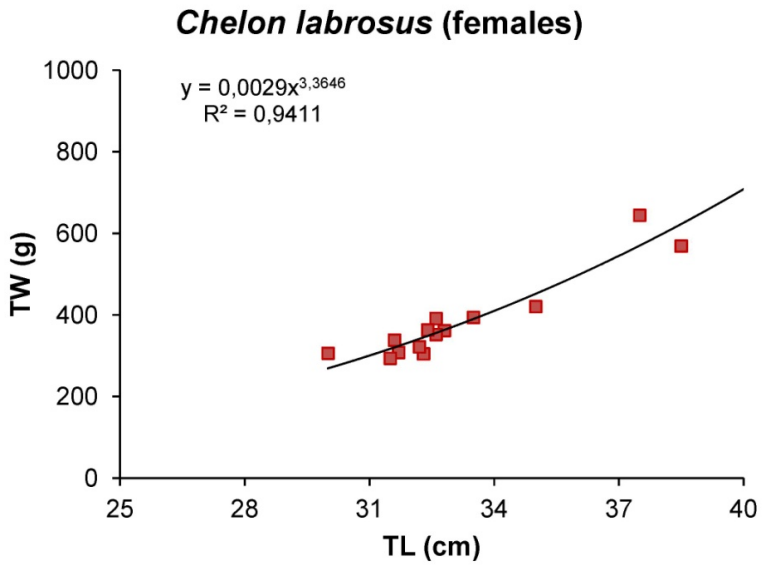


Fig. 2.26 Length-weight relationship of *Chelon labrosus* at Calich lagoon.

Liza aurata: 5 specimens (TL=32.0±7.7 cm; TW=295.4±219.9 g), of which 4 were

females (TL=33.7±7.8 cm; TW=335.6±231.7 g) only 1 was a male (TL=25.4 cm; TW=134.7 g).

Liza ramada: 93 specimens (TL=32.3± 2.4 cm; TW=304,0±65.9 g), of which 83 were females (TL=32.3±2.2 cm; TW=306.3±65.6 g; Fig. 2.27 & 2.28), 9 were males (TL=32.2±3.6 cm; TW=289.8±71.3 g; Figs. 2.29 & 2.30) and 1 was classified as intersex (TL= 30.1 cm; TW= 242.4 g). *Mugil cephalus*: 2 specimens, of which 1 was female (TL=33.2 cm; TW=403.0 g) and 1 was male (TL=30.4 cm; TW=290.4 g).

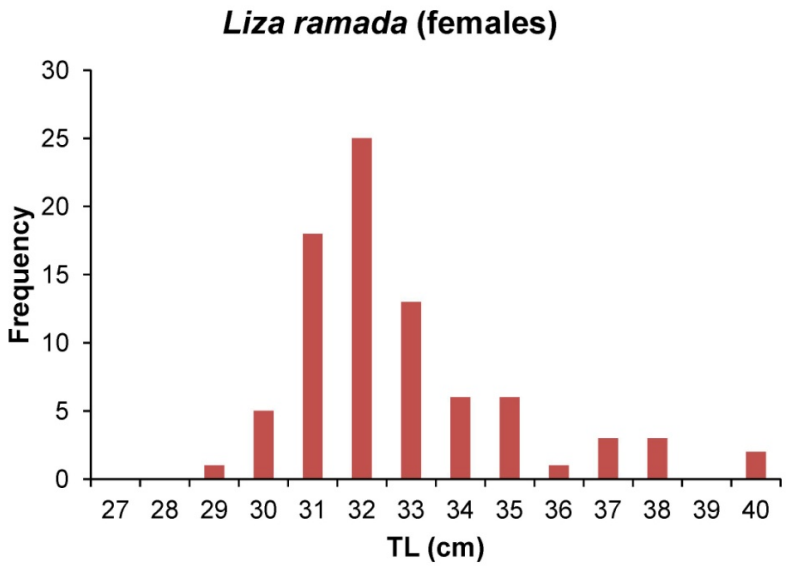


Fig. 2.27 Distribution of length size classes of *Liza ramada* at Calich lagoon.

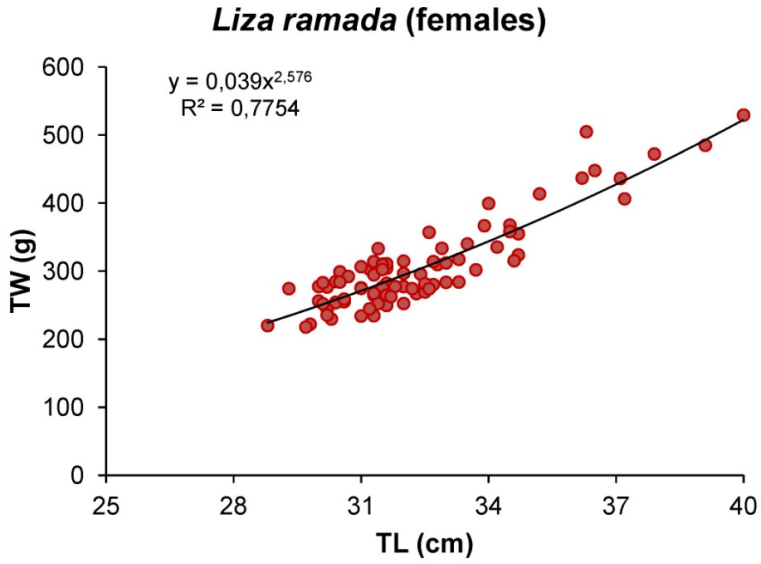


Fig. 2.28 Length-weight relationship of *Liza ramada* at Calich lagoon.

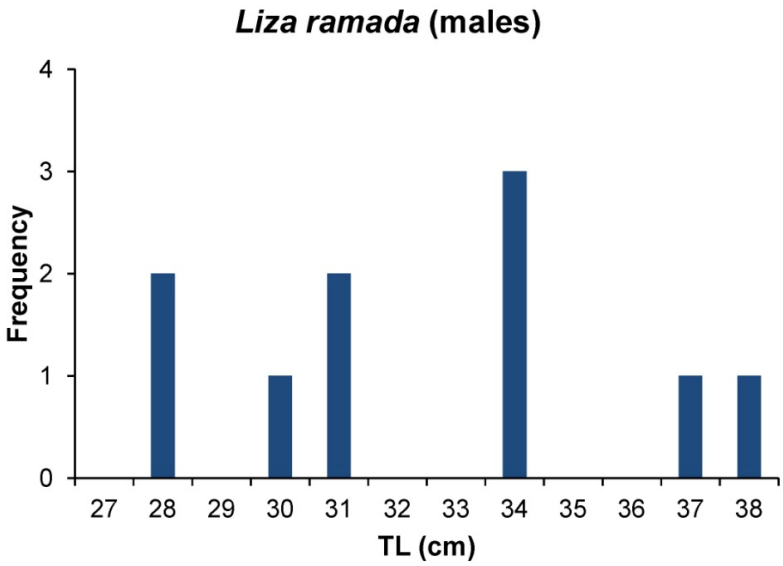


Fig. 2.29 Distribution of length size classes of *Liza ramada* at Calich lagoon.

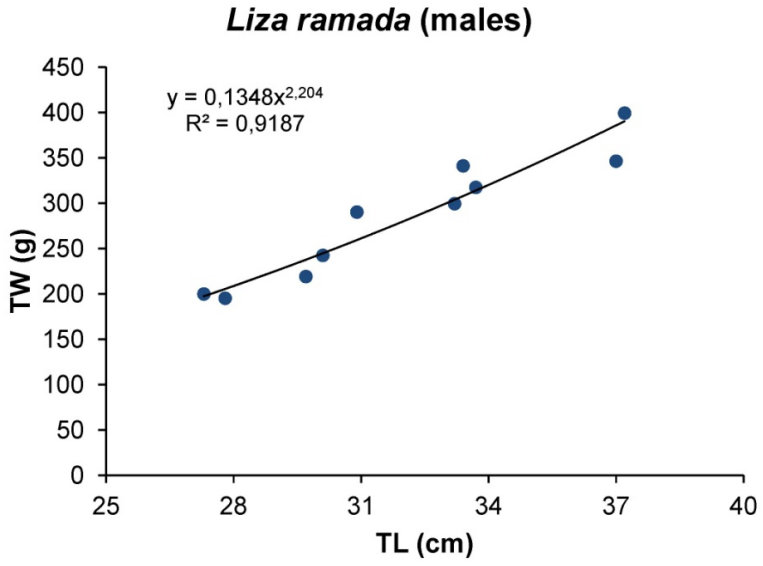


Fig. 2.30 Length-weight relationship of *Liza ramada* at Calich lagoon.

2.7.3 Marceddì

In total, 89 mullets were examined. *Chelon labrosus*: 8 specimens (TL=35.0±2.8 cm; TW=505.2±122.3 g), of which 6 were females (TL=33.9±1.9 cm; TW=458.3±74.7 g), 1 was a male (TL=36.0 cm; TW=536.8 g), and 1 was classified as intersex (TL=40.4 cm; TW=755.3 g).

Liza aurata: 74 specimens (TL=30.5±2.5 cm; TW=275.9±78.4 g), of which 61 were females (TL=30.9±2.5 cm; TW=286.5±80.6 g; Figs 2.31 & 2.32), 12 were males (TL=28.7±1.8 cm; TW=226.4±42.6 g; Figs. 2.33 & 2.34), and 1 was classified as intersex (TL=27.7 cm; TW=224.0 g).

Liza ramada: 2 female specimens (TL=37.1 and 31.6 cm, respectively; TW=477.0 and 309.6 g, respectively).

Mugil cephalus: 5 specimens (TL=36.6±5.9 cm; TW=566.1±202.4 g), of which 1 was a female (TL=39.9 cm; TW=686.5 g), 3 were males (TL=34.9±7.5 cm; TW=524.2±268.3 g), and 1 was classified as intersex (TL=38.7 cm; TW=571.6 g).

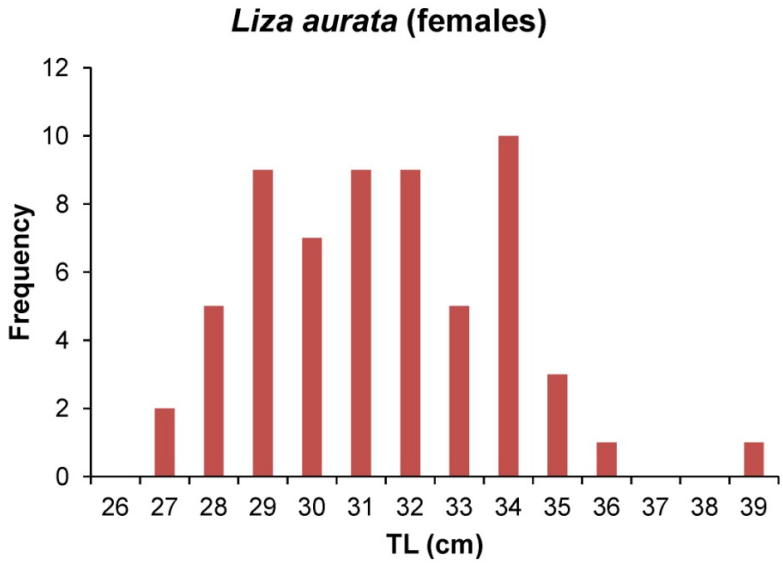


Fig. 2.31 Distribution of length size classes of *Liza aurata* at Marceddì lagoon.

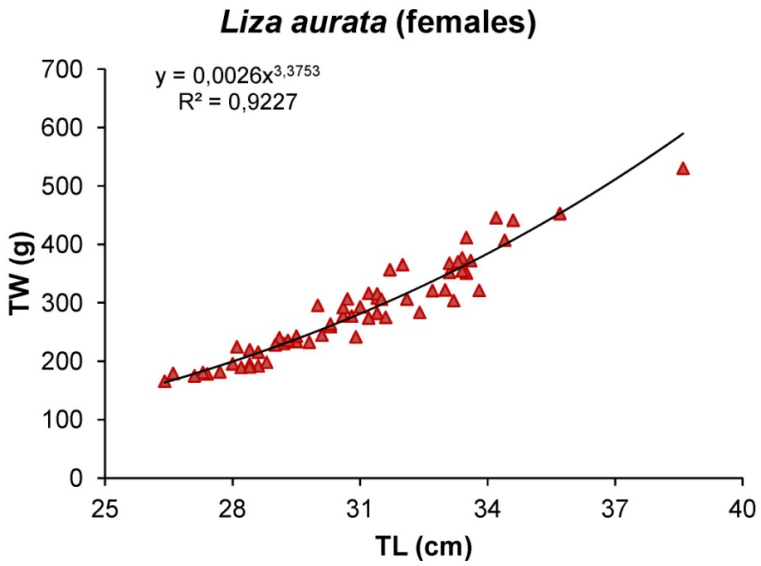


Fig. 2.32 Length-weight relationship of *Liza aurata* at Marceddì lagoon.

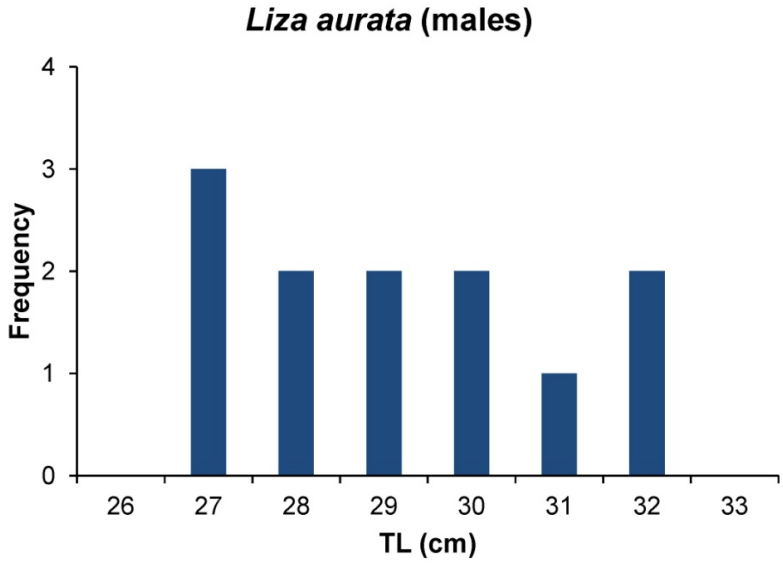


Fig. 2.33 Distribution of length size classes of *Liza aurata* at Marceddì lagoon.

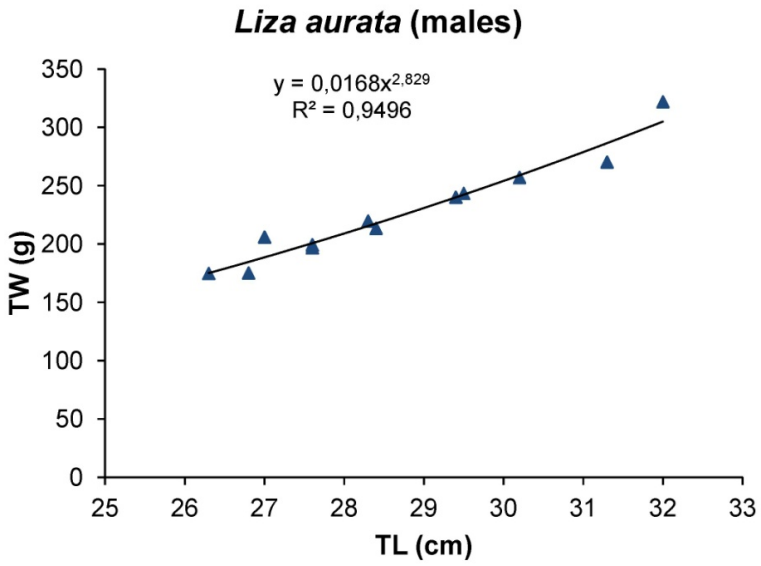


Fig. 2.34 Length-weight relationship of *Liza aurata* at Marceddì lagoon.

2.7.4 San Teodoro

Overall, 138 mullets were examined. *Chelon labrosus*: 26 specimens (TL=32.4±6.0 cm; TW=360.5±215.4 g), of which 13 were females (TL=32.6±7.0 cm; TW=380.0±241.5 g; Figs. 2.35 & 2.36), 8 were males (TL=33.6±5.4 cm; TW=388.3±230.6 g), and 5 were classified as intersex (TL=29.7±3.3 cm; TW=265.3±87.8 g).

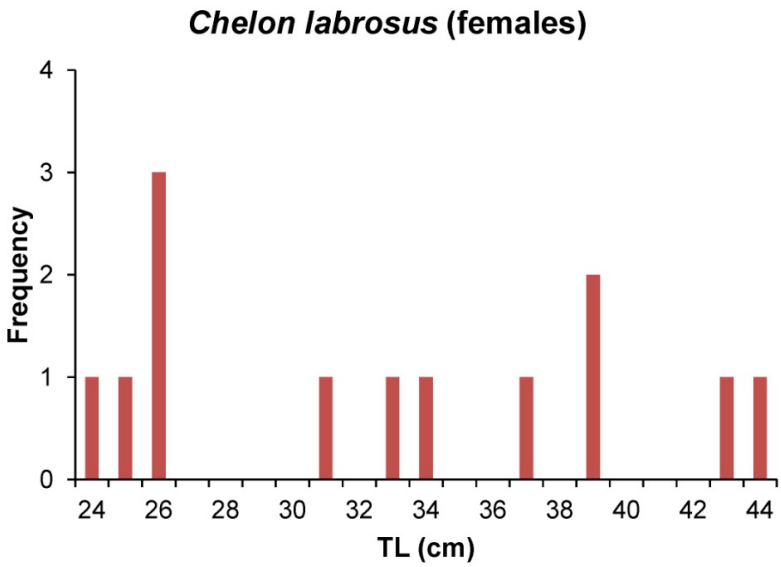


Fig. 2.35 Distribution of length size classes of *Chelon labrosus* at San Teodoro lagoon.

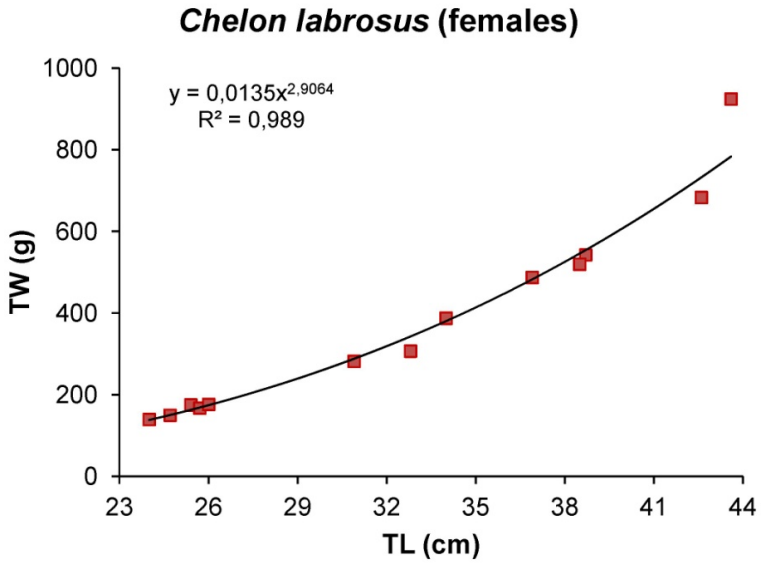


Fig. 2.36 Length-weight relationship of *Chelon labrosus* at San Teodoro lagoon.

Liza aurata: 62 specimens (TL=29.1±3.9 cm; TW=227.1±100.5 g), of which 44 were females (TL=30.0±4.1 cm; TW=248.5±109.6 g; Figs. 2.37 & 2.38), 16 were males (TL=26.8±1.8 cm; TW=177.5±41.5 g; Figs. 2.39 & 2.40), and 2 were classified as intersex (TL=24.27 and 28.7 cm, respectively; TW=109.0 and 196.2 g, respectively).

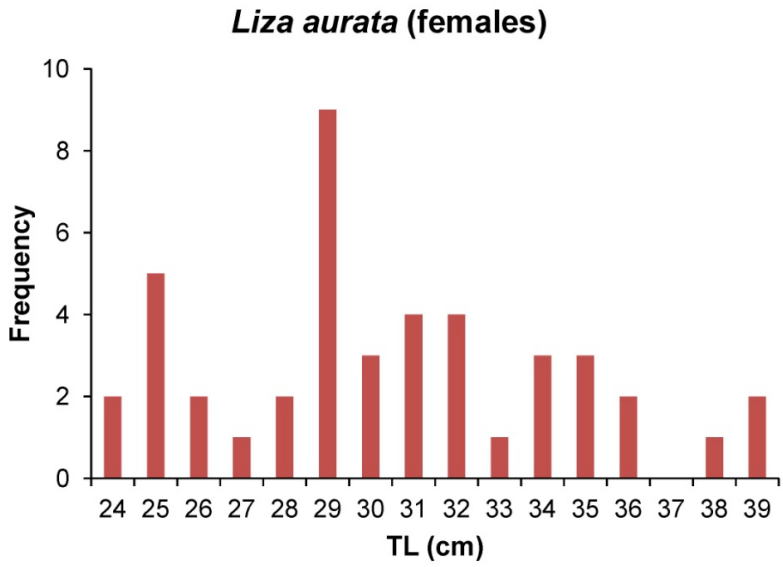


Fig. 2.37 Distribution of length size classes of *Liza aurata* at San Teodoro lagoon.

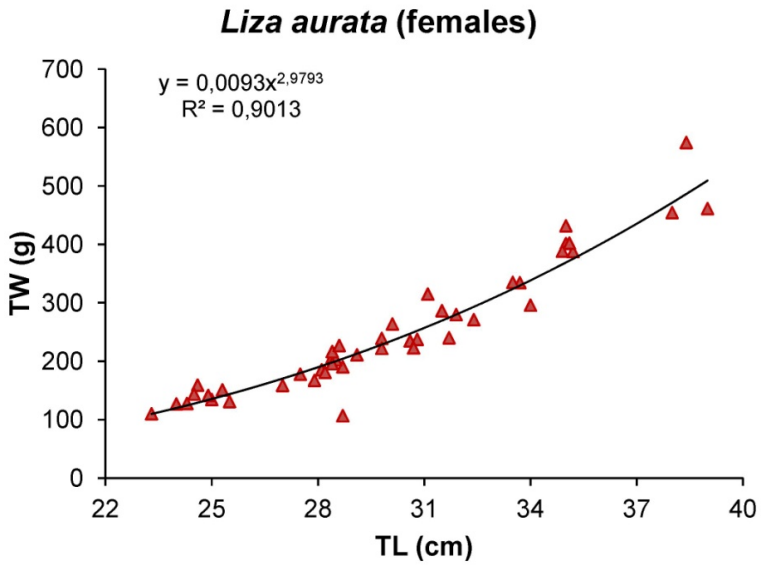


Fig. 2.38 Length-weight relationship of *Liza aurata* at San Teodoro lagoon.

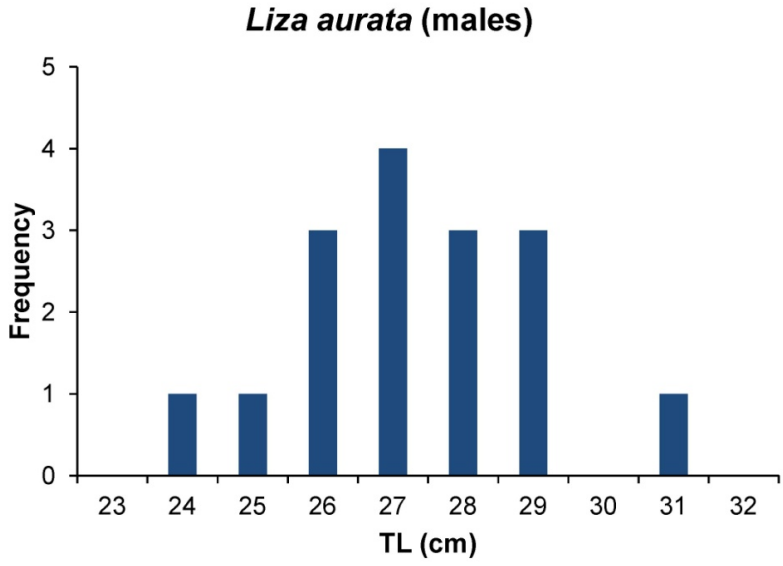


Fig. 2.39 Distribution of length size classes of *Liza aurata* at San Teodoro lagoon.

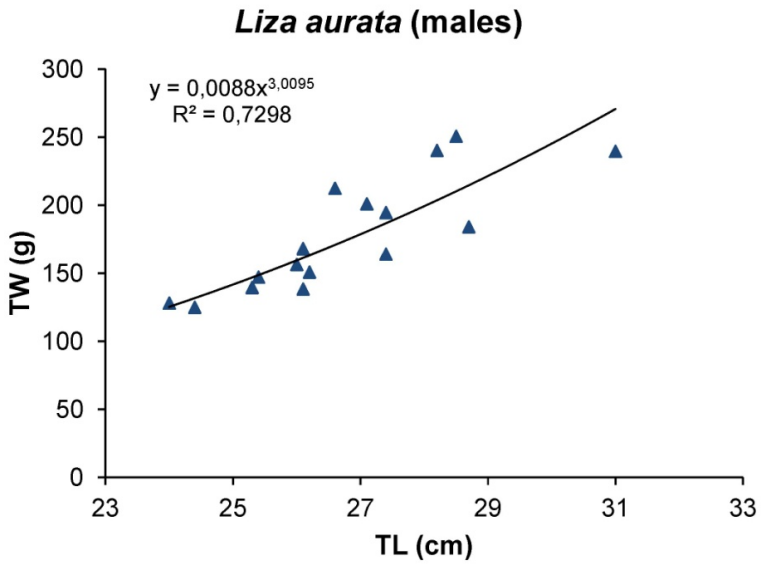


Fig. 2.40 Length-weight relationship of *Liza aurata* at San Teodoro lagoon.

Liza ramada: 9 specimens (TL=34.1±2.1 cm; TW=365.6±87.7 g), of which 6 were females (TL=34.4±1.8 cm; TW=373.7±83.9 g) and 3 were males (TL=33.4±3.0 cm; TW=349.6±112.3 g).

Mugil cephalus: 41 specimens (TL=45.7±8.1 cm; TW=1096.1±643.8 g) of which 15 were females (TL=50.1±8.7 cm; TW=1500.2±835.1 g; Figs. 2.41 & 2.42), 24 were males (TL=43.2±6.9 cm; TW=855.2±354.6 g; Figs. 2.43 & 2.44), and 2 were classified as intersex (TL=46.2 and 41.6 cm, respectively; TW=1101.2 and 812.0 g, respectively).

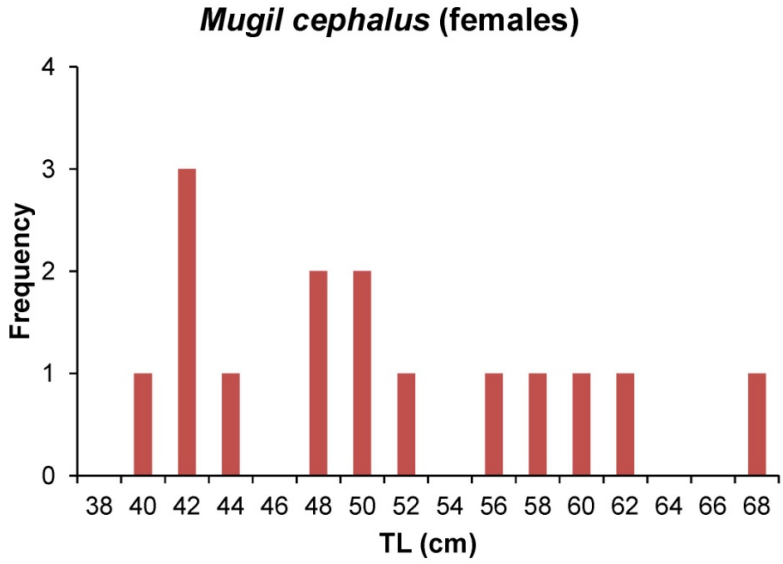


Fig. 2.41 Distribution of length size classes of *Mugil cephalus* at San Teodoro lagoon.

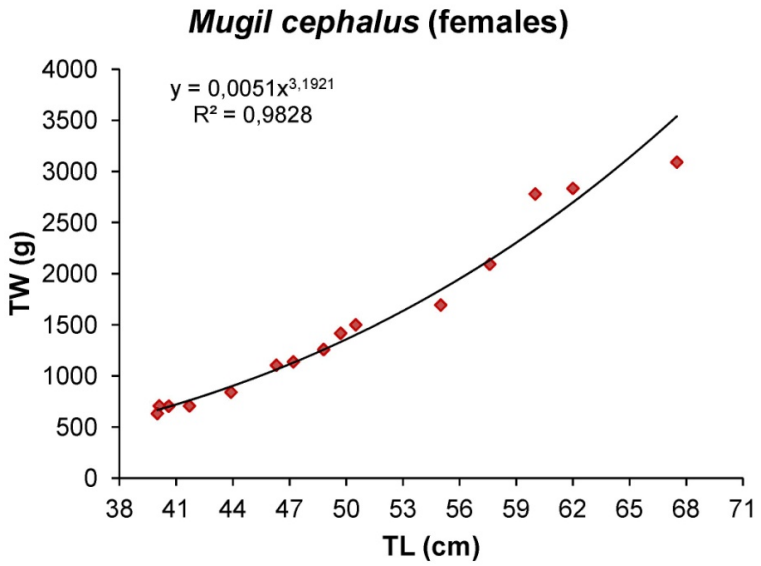


Fig. 2.42 Length-weight relationship of *Mugil cephalus* at San Teodoro lagoon.

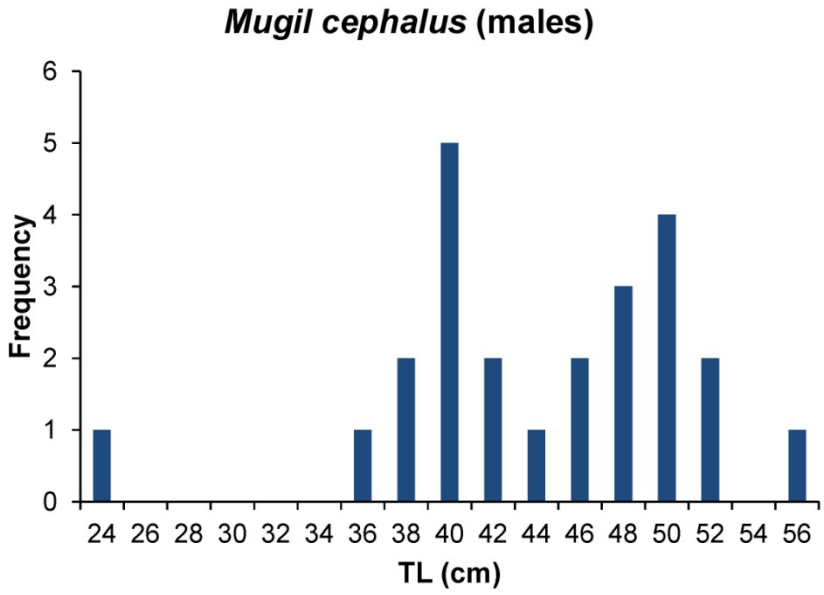


Fig. 2.43 Distribution of length size classes of *Mugil cephalus* at San Teodoro lagoon.

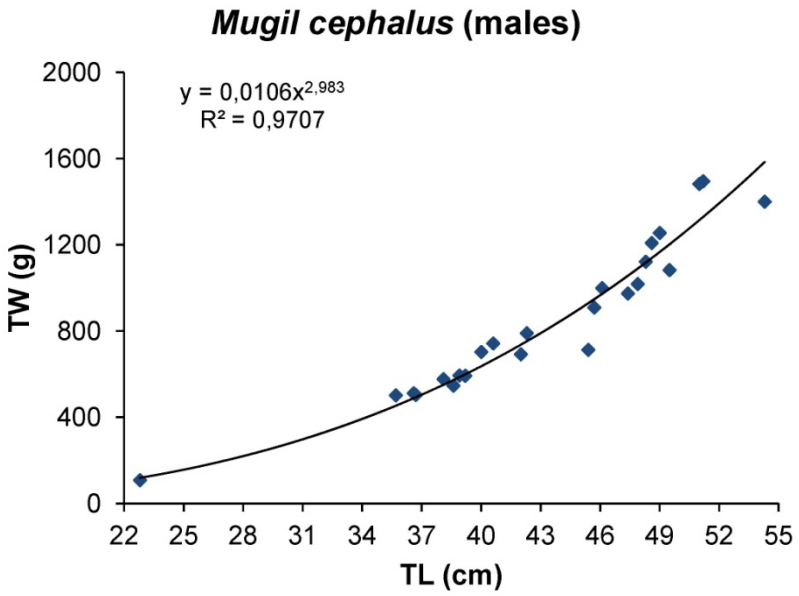


Fig. 2.44 Length-weight relationship of *Mugil cephalus* at San Teodoro lagoon.

2.7.5 Morphometric indices features

As far as morphometric indices is concerned, the results (mean±SD) of Fulton's Condition Factor (K), Epatosomatic Index and Gonadosomatic Index for all the Mugilidae species sampled at the four investigated lagoons are summarized in Tables 2.3, 2.4, 2.5 and 2.6.

Table 2.3 Morphometric indices of 144 *Liza ramada* and 4 *Mugil cephalus* specimens sampled at the Cabras lagoon.

Liza ramada	Females	Males
specimens	65	79
Fulton's Condition Factor (K)	0.87±0.79	0.88±0.08
Epatosomatic Index	1.03±0.27	1.01±0.21
Gonadosomatic Index	0.85±1.49	1.23±1.70
Mugil cephalus	Females	Males
specimens	1	3
Fulton's Condition Factor (K)	1.06	1.01±0.57
Epatosomatic Index	1.58	1.91±0.42
Gonadosomatic Index	0.56	0.13±0.08

Table 2.4 Morphometric indices of 20 *Chelon labrosus*, 5 *Liza aurata*, 93 *Liza ramada* and 2 *Mugil cephalus* specimens sampled at the Calich lagoon.

Chelon labrosus	Females	Males	Intersex
specimens	15	4	1
Fulton's Condition Factor (K)	1.04±0.09	1.00±0.09	0.96
Epatosomatic Index	1.57±0.22	1.52±0.37	1.00
Gonadosomatic Index	0.26±0.09	0.14±0.15	0.04
Liza aurata	Females	Males	Intersex
specimens	4	1	-
Fulton's Condition Factor (K)	0.80±0.05	0.82	-
Epatosomatic Index	1.22±0.37	0.98	-
Gonadosomatic Index	0.59±0.36	0.15	-
Liza ramada	Females	Males	Intersex
specimens	83	9	1
Fulton's Condition Factor (K)	0.90±0.09	0.86±0.09	0.89
Epatosomatic Index	1.32±0.32	1.18±0.37	1.72
Gonadosomatic Index	4.02±5.22	1.50±2.06	0.12
Mugil cephalus	Females	Males	Intersex
specimens	1	1	-
Fulton's Condition Factor (K)	1.01	1.03	-
Epatosomatic Index	1.41	1.47	-
Gonadosomatic Index	0.17	0.07	-

Table 2.5 Morphometric indices of 8 *Chelon labrosus*, 74 *Liza aurata*, 2 *Liza ramada* and 5 *Mugil cephalus* specimens sampled at the Marceddì lagoon.

Chelon labrosus	Females	Males	Intersex
specimens	6	1	1
Fulton's Condition Factor (K)	1.17±0.06	1.15	1.15
Epatosomatic Index	1.91±0.32	1.86	2.01
Gonadosomatic Index	0.21±0.04	0.11	0.16
Liza aurata	Females	Males	Intersex
specimens	61	12	1
Fulton's Condition Factor (K)	0.95±0.08	0.95±0.04	1.05
Epatosomatic Index	1.71±0.68	1.45±0.36	1.54
Gonadosomatic Index	2.88±3.67	0.62±1.01	0.05
Liza ramada	Females	Males	Intersex
specimens	2	-	-
Fulton's Condition Factor (K)	0.96±0.03	-	-
Epatosomatic Index	1.70±0.06	-	-
Gonadosomatic Index	0.87±0.25	-	-
Mugil cephalus	Females	Males	Intersex
specimens	1	3	1
Fulton's Condition Factor (K)	1.08	1.15±0.04	0.99
Epatosomatic Index	1.61	2.14±0.29	1.35
Gonadosomatic Index	0.32	0.05±0.00	0.23

Table 2.6 Morphometric indices of 26 *Chelon labrosus*, 62 *Liza aurata*, 9 *Liza ramada* and 41 *Mugil cephalus* specimens sampled at the San Teodoro lagoon.

Chelon labrosus	Females	Males	Intersex
specimens	13	8	5
Fulton's Condition Factor (K)	0.98±0.07	0.95±0.07	0.98±0.05
Epatosomatic Index	1.12±0.25	1.25±0.15	1.19±0.07
Gonadosomatic Index	0.29±0.20	0.07±0.03	0.05±0.02
Liza aurata	Females	Males	Intersex
specimens	44	16	2
Fulton's Condition Factor (K)	0.87±0.10	0.91±0.11	0.80±0.04
Epatosomatic Index	1.40±0.39	1.46±0.56	1.52±0.10
Gonadosomatic Index	2.74±4.16	1.06±0.87	0.07±0.03
Liza ramada	Females	Males	Intersex
specimens	6	3	-
Fulton's Condition Factor (K)	0.90±0.08	0.92±0.10	-
Epatosomatic Index	1.65±0.39	1.20±0.39	-
Gonadosomatic Index	1.30±0.96	0.05±0.04	-
Mugil cephalus	Females	Males	Intersex
specimens	15	24	2
Fulton's Condition Factor (K)	1.09±0.09	1.00±0.09	1.12±0.01
Epatosomatic Index	1.91±0.31	2.03±0.32	2.08±0.11
Gonadosomatic Index	0.74±0.64	0.79±1.32	0.28±0.33

All the above mentioned Mugilidae species (i.e. *Chelon labrosus*, *Liza aurata*, *Liza ramada* and *Mugil cephalus*) sampled at the 4 Sardinian lagoons (i.e. Cabras, Calich, Marceddì and San Teodoro) were subsequently examined for mycobacteriosis (see Chapter 3) and gonadal anomalies and/or presence of intersex condition (see Chapter 4).

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Chapter 3

MYCOBACTERIOSIS CAUSED BY ATYPICAL MYCOBACTERIA IN REARED MULLETS: FIRST EVIDENCE FROM SARDINIA

3.1 Introduction

Mycobacteriosis is a chronic progressive disease caused by several acid-fast bacteria of the genus *Mycobacterium* affecting wild and cultured fish worldwide (Jacobs *et al.*, 2009). *Mycobacterium chelonae*, *M. fortuitum* and *M. marinum* are the most common etiologic agents of this pathology, also called "piscine tuberculosis". These atypical mycobacteria are saprophytes both in soil and in waters, where they can live for years. In particular, *M. marinum* is a bacillus ubiquitous in nature, forming the largest portion of all mycobacteria isolated from fish (Decostere *et al.,* 2004). Although various routes of infection are possible in fish, oral transmission through feces or carcasses of infected fish are recognized as the primary cause of mycobacteriosis (Ackleh *et al.,* 2014). Furthemore, it has been demonstrated that amoebae or *Paramecium caudatum* are usually required for efficient transmission of *M. marinum* in some species (e.g. zebrafish: Harriff *et al.,* 2007; Peterson *et al.,* 2013).

In fish mycobacteriosis, gross examination may reveal greyish to white granulomatous nodules in various organs (mainly in spleen, liver and kidney) (Zanoni *et al.,* 2008), although clinical signs are often lacking and hardly detectable until advanced stages. Diagnosis of this disease is based on the detection of granulomas and acid-fast bacteria in tissue sections, generally visible with Ziehl-Neelsen stain (Gauthier & Rhodes, 2009). Microscopically, typical granulomas show peripheral fibrosis and a number of layers of epithelioid cells surrounding a necrotic core with a variable amount of acid-fast bacilli (Astrofsky *et al.,* 2000; Decostere *et al.,* 2004). Nevertheless, as experimentally observed by Colorni *et al.* (1998) and recently also by Ortega *et al.* (2014), granulomas can be also histologically classified in distinct evolutionary stages.

In general, acid-fast bacilli are frequently visible within granulomas, although granulomas with no visible acid-fast bacilli have been observed in a number of experimentally infected fish (Gauthier & Rhodes, 2009 and references therein), probably depending on several factors like species, abundance and growth stage of the pathogen (Jacobs *et al.,* 2009).

On the other hand, it is worth mentioning that several species of Actinomycetes are partially acid-fast, and *Legionella* spp. occasionally demonstrates some acid-fastness. In any case, immunohistochemical technique may be useful for revealing mycobacteria in early granulomas (Gauthier & Rhodes, 2009 and references therein).

For a final diagnosis of mycobacteriosis, however, isolation and identification of the microorganisms are needed. At this scope, specific media and phenotypical tests,

including fatty acid and mycolic acid analysis, are used. Subsequently, classification should be definitely validated by 16S ribosomal gene DNA sequencing (Toranzo *et al.*, 2005). All fish should be considered vulnerable to mycobacteriosis. In fact, more than 150 freshwater and marine fish species are known to be affected by this disease (Chinabut, 1999), with a higher occurrence in cultured species (Gauthier & Rhodes, 2009). The effect of this disease in aquaculture and in ornamental fish industry has been well documented by several authors (Prearo *et al.,* 2002; Lescenko *et al.,* 2003; Prearo *et al.,* 2004; Pate *et al.,* 2005; Beran *et al.,* 2006; Zanoni *et al.,* 2008; Antuofermo *et al.,* 2014; Favaro *et al.,* 2014; Righetti *et al.,* 2014) as well as the capability of different species of *Mycobacterium* to infect humans causing granulomatous skin lesions (Petrini, 2006). However, because *Mycobacterium marinum* is one of the main fish pathogen described in association to zoonotic disease, the ability to distinguish among different strains remains of great importance (Haenen *et al.,* 2013).

The Mugilidae family includes coastal marine and brackish water species distributed in all temperate and tropical seas (Nelson, 2006). These fishes are of economic importance for fisheries and aquaculture purposes as they are a major food source in several regions of the world. At present, acid-fast bacterial infections in wild Mugilidae are scarcely reported worldwide: Osman (1980) observed several cases in *Liza aurata* from Libya; Rodrigues & Fernandes de Araujo (1983) in a single *Mugil curema* from Brazil; Couch (1985) in an adult of *Mugil cephalus* from the Gulf of Mexico; and, more recently, Varello *et al.* (2014) in a number of species from Italy. Furthermore, fish mycobacteriosis was also detected in cultured mullets (Perez *et al.*, 2001; Salati *et al.*, 2010).

The aim of the present work was to investigate the occurrence of mycobacteriosis in visceral organs of several species of cultured Mugilidae from four lagoons of Sardinia (Italy) devoted to extensive aquaculture. At this scope, different diagnostic methods for detection of mycobacteria were performed (acid-fast stained histological sections, plate culture, PCR-RFLP and sequencing).

3.2 Material and methods

3.2.1 Study area

Lagoons and coastal ponds of Sardinia cover an area of more than 15.000 hectares. In most of these brackish environments extensive aquaculture is practiced and mullets are cultured among other euryhaline fish species (Cannas *et al.,* 1998).

In this study, four of these wetlands were examined: the Cabras and the Marceddì lagoons (Central western Sardinia) the Calich lagoon (North western Sardinia) and the San Teodoro lagoon (North eastern Sardinia). For a detailed description of the sites and lagoon characteristics see Fig 2.1 and Chapter 2.

3.2.2 Sampling

Four hundred and ninety-five adult specimens belonging to the family Mugilidae (*Chelon labrosus*, *Liza aurata*, *Liza ramada* and *Mugil cephalus*) were sampled twice a year from July 2013 to November 2015. For detailed characteristics of the Mugilidae species considered and their sampling see Chapter 2.

3.2.3 Anatomo-histopathological evaluation

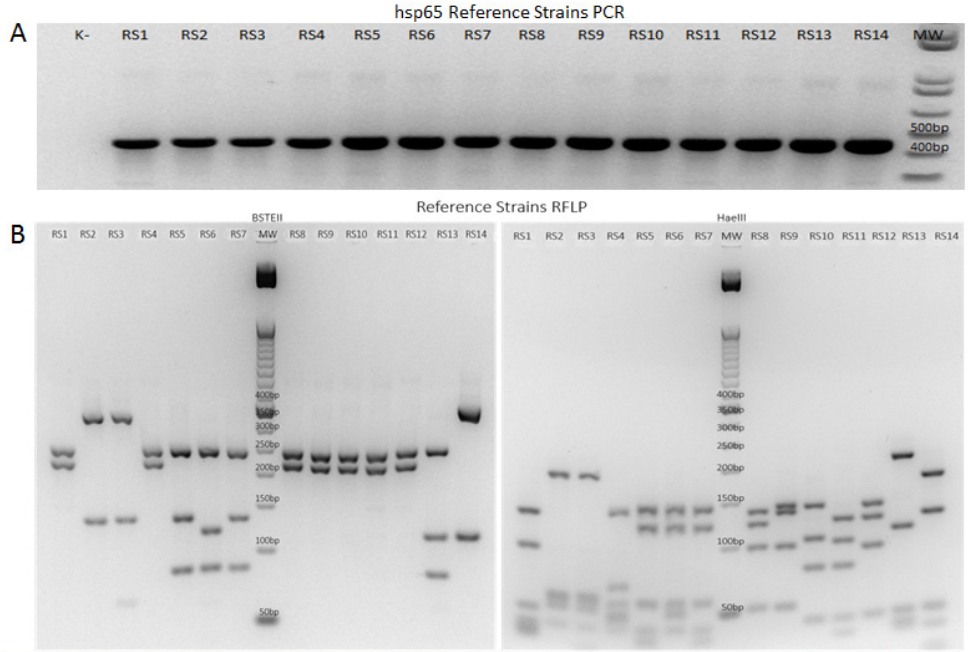
All the fish were classified at species level following Farrugio (1977), weighed, measured, and photographed. Subsequently, necropsy and gross examination of each subject was performed and samples of target visceral organs (i.e. hearth, kidney, liver and spleen) were fixed in 10% neutral formalin and microscopically evaluated as reported in detail in Chapter 2. Additionally samples of liver and spleen were stored at -20° C and then sent to the laboratory of fish diseases of the "Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta" (Torino), in order to perform bacteriological analyses. Moreover, extra samples of spleen were stored at -80°C and processed in our laboratory for molecular biology analyses.

3.2.4 Bacteriological and molecular analyses

Fresh tissues from spleen and liver were homogenized and decontaminated for 30 minutes using 1.5% cetylpyridinium chloride monohydrate (AppliChem). A volume of 10 μL of the homogenate was spread on a glass slide, over an area of approximately 1 × 2 cm, and then stained using the ZN method. At least 300 fields were microscopically examined at high magnification (×1000). For culture, one loop of 10 μL of the

decontaminated homogenate was inoculated on 2 Stonebrink medium tubes [Microbiol, Uta (CA), Italy] and 2 Löwenstein–Jensen medium tubes. One tube from each medium was incubated at 37±2°C and one tube at 28±2°C. The tubes were checked daily for 2 months; all suspected colonies were microscopically examined using ZN staining and were also subcultured.

All isolates were identified following the methods of Kent & Kubica (1985). A panel of the following 14 mycobacteria reference strains were used for the development of the biomolecular assays: *Mycobacterium abscessus* (DSM 44196), *M. chelonae* (DSM 43804), *M. flavescens* (DSM 43991), *M. fortuitum* (DSM 46621), *M. gordonae* (DSM 43212), *M. interjectum* (DSM 44064), *M. kansasii* (DSM 44162), *M. marinum* (DSM 44344), *M. peregrinum* (DSM 43271), *M. salmoniphilum* (DSM 43276), *M. scrofulaceum* (DSM 43992), *M. septicum* (DSM 44393), *M. smegmatis* (DSM 43756), and *M. terrae* (DSM 43227) (Fig. 3.1).



C

	Reference strains	BSTEII pattern	HaeIII pattern		Reference strains	BSTEII pattern	HaeIII pattern
RS1	*M. flavescens* (DSMZ 43991-ATCC 14474)	235-210	140-110-50	RS8	*M. septicum* (DSMZ 44393-ATCC 700731)	230-210	130-120-105-60
RS2	*M. chelonae* (DSMZ 43804-ATCC 35752)	320-130	180-65-55	RS9	*M. peregrinum* (DSMZ 43271-ATCC 14467)	230-210	140-130-105-60
RS3	*M. salmoniphilum* (DSMZ 43276-ATCC 13758)	320-130	180-65-55	RS10	*M. marinum* (DSMZ 44344-ATCC 927)	230-210	140-110-80
RS4	*M. abscessus* (DSMZ 44196-ATCC19977)	235-210	140-70-60-50	RS11	*M. kansasii* (DSMZ 44162-ATCC 12478)	230-210	140-110-80
RS5	*M. interjectum* (DSMZ 44064-ATCC 51457)	230-130-80	140-120-60-55	RS12	*M. scrofulaceum* (DSMZ 43992-ATCC 19981)	230-210	150-130-100
RS6	*M. fortuitum* (DSMZ 46621-ATCC6841)	230-125-80	140-120-60	RS13	*M. gordonae* (DSMZ 43212-ATCC 35756)	235-130-80	230-135
RS7	*M. smegmatis* (DSMZ 43756-ATCC 19420)	230-130-80	140-120-60	RS14	*M. terrae* (DSMZ 43227-ATCC 157755)	310-130	190-140-60

Fig. 3.1 Reference strains. Hsp65 PCR amplicons of the reference strains (A), PRA analysis (B), and legend of PRA pattern for each strain (C).

DNA extraction was performed from 25 mg of spleen, collected from each fish and

stored at -20°C, using DNeasy® Blood & Tissue (Qiagen) according to the manufacturer's instructions. The amplification of a 441 bp fragment of the hsp65 gene was performed using primers Tb11 (5'-ACCAACGATGGTGTGTCCAT) and Tb12 (5'-CTTGTCGAACCGCATACCCT) as described by Telenti *et al.* (1993). The PCR reaction mix contained 1× reaction buffer (with 1.5 mM MgCl2), 1 × CoralLoad, 200 mM dNTP, 25 pmoles of each primer and 1U TopTaq DNA polymerase (Qiagen). PCR amplification consisted of an initial denaturation at 93°C for 10 min, followed by 45 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were analyzed by 2% agarose gel electrophoresis. RFLP was carried out using BstEII and HaeIII restriction enzymes. Ten microliters of each PCR product were digested, separately, with 5 U of BstEII and HaeIII for 2 h at 37°C followed by a deactivation of the enzymes at 80°C for 20 min. The digested products were loaded on 4% agarose gel electrophoresis at 100 V for 2 h. To interpret the PRA profiles generated by each sample, a 50 bp ladder DNA size marker (50 bp ladder-Invitrogen) was used. The fragments were detected with GelRed staining and UV transilluminator. Restriction patterns of the reference strains and of our samples were estimated using the PRASITE DATABASE query (http://app.chuv.ch/prasite/index.html). An aliquot of the 441 bp PCR products were purified, using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, and subjected to direct sequencing. The sequencing was performed with the Sanger method, on an ABI PRISM sequencing apparatus (ABPRISM 3500 Genetic Analyser, Applied Biosystems) using Big Dye Terminator kit (Applied Biosystems) that includes didesoxynucleotides marked with four fluorochromes of different colours. For each PCR product, both strands were sequenced, in independent reactions, using Tb11 or Tb12 primers. The resulting electropherograms were manually edited to ensure sequence accuracy, using BioEdit v. 7.2.5 (Hall, 1999), then the sequences were entered into the Basic Local Alignment and Research Tool (BLAST) in GenBank (https://www.ncbi.nlm.nih.gov/) to search highly similar sequences. The phylogenetic analysis was performed using MEGA v.6 software package (Tamura *et al.,* 2013).

The phylogenetic tree was obtained using Neighbor-Joining method, and the evolutionary distances were computed using Kimura 2-parameter model and 1000 bootstrap replications.

3.3 Results

3.3.1 Macro and microscopical findings

Grossly, internal lesions (i.e. focal small white nodules ranging from 0.2 to 0.5 mm) were found in 11 out of the 25 mullet specimens affected by mycobacteriosis. The most affected organs were spleen (9/25) and liver (5/25) (Fig. 3.2). No gross lesions were instead observed in heart and kidney.

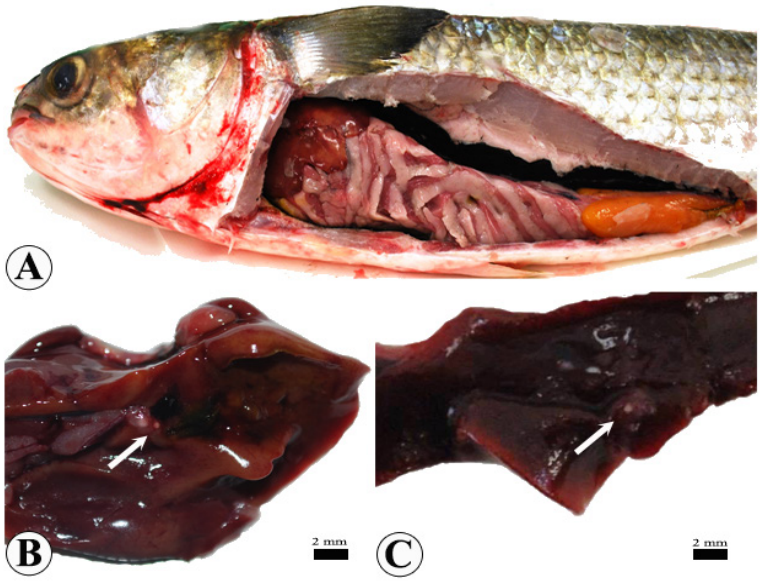


Fig. 3.2 *Mugil cephalus.* Internal organs affected by mycobacteriosis (A). Multifocal small whitish nodules ranging in size from 0.2-0.5 cm (arrows) in liver (B) and spleen (C) (bar=2 mm).

Histologically, 25 mullets showed ZN positive granulomas for acid-fast bacteria. In detail, 2 specimens of *Liza ramada* were fished in the Cabras lagoon (C8 and C27); 4 *Liza aurata* (STA11, STA14, STA15 and STA134), 3 *Chelon labrosus* (STA26, STA29 and STA86); 3 *Mugil cephalus* (STA33, STA113 and STA120) and 1 *L. ramada* (STA40) from the San Teodoro lagoon; 1 *L. aurata* (CH94), 3 *C. labrosus* (CH15, CH66, CH93), 8 *L. ramada* (CH23, CH24, CH43, CH150, CH52, CH56, CH67, CH120) from the Calich lagoon. Six specimens showed two or more affected organs, whereas 19 had granulomas in only one organ. Spleen was the most affected organ (23 specimens), followed by liver (7 specimens) and heart (3 specimens). No granulomas were observed in kidney (Table 3.1).

Table 3.1 Atypical micobacteria identification by multiple approaches. Granulomas in mullet organs positive to Ziehl-Neelsen (ZN), and hsp65 PCR-RFLP characterization. *Mycobacterium* bacteriological isolation, and sequencing identification. (Cn=Cabras; STAn=San Teodoro; CHn=Calich; TL=Total length).

Case #	ID #	Species	Seasons	Sex	TL	Organ	Macro	ZN	Microbiology	PCR-RFLP	Sequencing
1	C8	*L. ramada*	summer 2013	female	34.5	heart	-	+	-	-	-
2	C27	*L. ramada*	summer 2013	male	29.2	liver	-	+	-	-	-
						spleen	-	+	*M. marinum*	*M. marinum*	*M. marinum*
3	STA11	*L. aurata*	summer 2013	female	30.6	liver	+	+	-	-	-
						spleen	+	+	*M. marinum*	*M. marinum*	*M. marinum*
4	STA14	*L. aurata*	summer 2013	female	31.7	spleen	+	+	-	-	-
5	STA15	*L. aurata*	summer 2013	female	32.4	heart	-	+	-	-	-
						liver	+	+	*M. marinum*	-	-
						spleen	+	+	*M. marinum*	*M. marinum*	*M. marinum*
6	STA26	*C. labrosus*	summer 2013	female	38.5	spleen	+	+	*Mycobacterium* spp.	-	-
7	STA29	*C. labrosus*	summer 2013	female	34.7	spleen	+	+	-	-	-
8	STA33	*M. cephalus*	autumn 2013	female	46.3	heart	-	+	-	-	-
						liver	+	+	-	-	-
						spleen	+	+	*M. marinum*	*M. marinum*	*M. marinum*
9	STA40	*L. ramada*	autumn 2013	female	33.6	spleen	-	+	*M. marinum*	*M. marinum*	*M. marinum*
10	CH15	*C. labrosus*	summer 2014	male	29.4	spleen	-	+	*Mycobacterium* spp.	*Mycobacterium* spp.	*Mycobacterium* spp.
11	CH23	*L. ramada*	summer 2014	female	32	spleen	+	+	-	*Mycobacterium* spp.	*Mycobacterium* spp.

Table 3.1 Continued.

Case #	ID #	Species	Seasons	Sex	TL	Organ	Macro	ZN	Microbiology	PCR-RFLP	Sequencing
12	CH24	*L. ramada*	summer 2014	female	30.4	spleen	-	+	-	-	-
13	CH43	*L. ramada*	summer 2014	female	36.2	liver	+	+	*Mycobacterium* spp.	-	-
						spleen	-	+	*Mycobacterium* spp.	-	-
14	CH50	*L. ramada*	summer 2014	female	33.7	spleen	-	+	-	*Mycobacterium* spp.	*Mycobacterium* spp.
15	CH52	*L. ramada*	summer 2014	female	32.8	spleen	-	+	-	-	-
16	STA86	*C. labrosus*	summer 2014	intersex	34.2	spleen	-	+	-	-	-
17	CH56	*L. ramada*	autumn 2014	female	35.7	spleen	-	+	-	*Mycobacterium* spp.	*Mycobacterium* spp.
18	CH66	*C. labrosus*	summer 2015	intersex	30.9	liver	+	+	*Mycobacterium* spp.	-	-
						spleen	-	+	*Mycobacterium* spp.	-	-
19	CH67	*L. ramada*	summer 2015	female	32.5	spleen	-	+	-	-	-
20	STA113	*M. cephalus*	summer 2015	male	49	spleen	-	+	*Mycobacterium* spp.	-	-
21	STA120	*M. cephalus*	summer 2015	male	40.6	spleen	+	+	*M. marinum*	*M. marinum*	*M. marinum*
22	STA134	*L. aurata*	summer 2015	female	39	spleen	-	+	*Mycobacterium* spp.	-	-
23	CH93	*C. labrosus*	autumn 2015	female	32.4	liver	-	+	*Mycobacterium* spp.	-	-
24	CH94	*L. aurata*	autumn 2015	female	44.9	spleen	+	+	-	-	-
25	CH120	*L. ramada*	autumn 2015	male	27.3	spleen	-	+	-	-	-

Based on histological pattern, granulomas were classified in 3 categories according to their evolutionary stage. Early stage granulomas were characterized only by macrophages aggregates. Intermediate stage granulomas were composed of macrophages surrounding a necrotic central core. In late stage granulomas, layers of fibroblasts delimited necrotic or laminar material.

Different amount of mycobacteria were detected in each evolutionary stage of observed granulomas (Fig. 3.3).

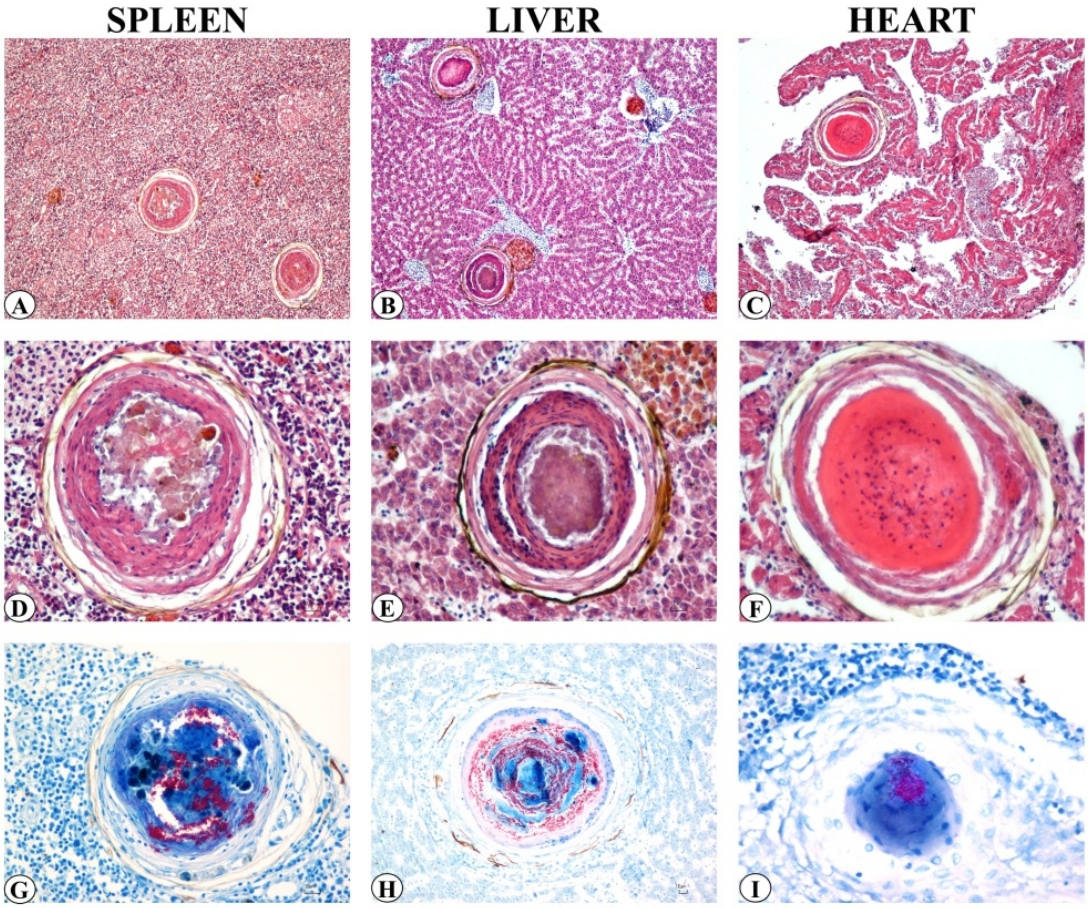


Fig. 3.3 Multiple granulomas throughout the splenic (A) and liver (B) parenchyma (HE stain, bar = 50 µm). (C) Focal granuloma in heart (HE stain, bar = 50 µm). D, E, and F: high magnification of A, B and C. Intermediate granulomas with focal central necrosis in spleen (D) and showing a central area of coagulative necrosis lined by a layer of flat cells and macrophages in liver (E) (HE stain, bar=100 µm). Late granuloma composed of laminar material without necrotic core within cardiac muscle (F) (HE stain, bar=100 µm). Numerous acid-fast rods are visible within spleen (G), liver (H) and heart (I) granulomas (ZN stain, bar=100 µm).

3.3.2 Bacteriological and molecular findings

Colonies have grown from cultured spleen and liver samples of 16 out of 495 (3%) mullets and were clearly visible in Löwenstein-Jensen and Stonebrink media tubes, showing acid-fast bacilli at ZN stain.

In particular, spleen samples were positive in 12 out of 23 of the subjects that showed ZN positive splenic granulomas (*Liza aurata*: STA11, STA15, STA134; *Liza ramada*: C27, STA40, CH43; *Chelon labrosus*: STA26, CH15, CH66; *Mugil cephalus*: STA33, STA113, STA120), while liver samples were positive in 4 out of 7 mullets that showed ZN positive liver granulomas (*L. aurata*: STA15; *L. ramada*: CH43; *C. labrosus*: CH66 and CH93). These colonies were phenotypically and biochemically identified as *Mycobacterium marinum* and *Mycobacterium* spp. (Table 3.1).

The results of PCR showed that 23 out of 495 spleen samples yielded amplicons of 441 bp. Ten samples yielded the restriction pattern of 245, 210 bp with BstEII and 144, 110, 80 bp with HaeIII, identifying species of mycobacteria.

In detail, 6 samples from San Teodoro (STA11, STA15, STA33, STA40, and STA120) and from Cabras (C27) showed a restriction pattern like *M. marinum* (Fig. 3.4). The remaining 4 samples from Calich (CH15, CH23, CH50 and CH56) showed restriction patterns comparable with *Mycobacterium* spp. (Fig. 3.5). The other 13 samples displayed patterns were different from all species of *Mycobacterium* spp. of the PRASITE database.

To definitively classify our samples, amplicons of 441 bp of all 23 specimens were sequenced. The analysis of sequences on GenBank database using ClustalW software showed 6 samples from San Teodoro (STA11, STA15, STA33, STA40, STA120, and C27) sharing >99% nucleotide identity with hsp65 sequences of *M. marinum* (Fig. 3.4).

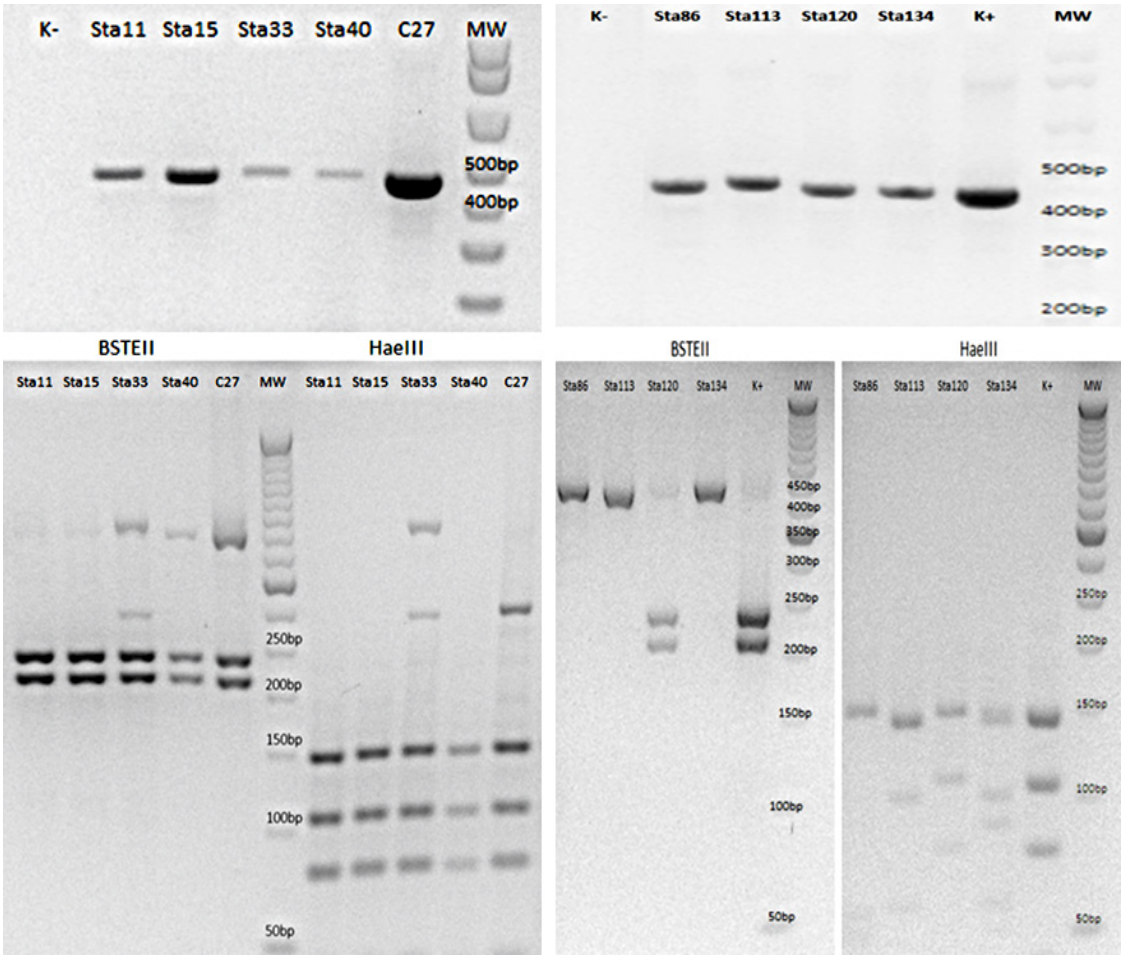


Fig. 3.4 Hsp65 amplicons (441 bp) and restriction patterns of the spleen samples from San Teodoro (Sta) and from Cabras (C) obtained using the enzymes BSTEII and HaeIII corresponding to *Mycobacterium marinum* (MW = 1 Kb plus and 50 bp DNA Ladder; K+ = positive control).

Four samples from Calich showed some similarity with different species of *Mycobacterium* [CH15 shared 98% of identity with *Mycobacterium* spp. (EU619905); CH23 96% with *M. genavense* (EU 495310); CH50 96% with *M. simulans* (FJ786253) and CH56 95% with *M. aurum*] (Fig. 3.5). The analysis of the alignment showed that the samples C27, STA11 and STA120 are very similar to *M. marinum* reference strain. They showed only one point mutation G/T in position 347, while STA15 showed 2 additional points mutations G/A in position 1 and C/A in position 8. STA40 an additional point mutation G/C in position 87. STA33 showed only one mutation T/C in position 205. All CH samples showed several differences with *M. marinum* (Fig. 3.6). This result is in agreement with the restriction patterns and with the percentage of identity detected on GenBank with other species of *Mycobacterium* spp. The alignment was obtained using ClustalW software.

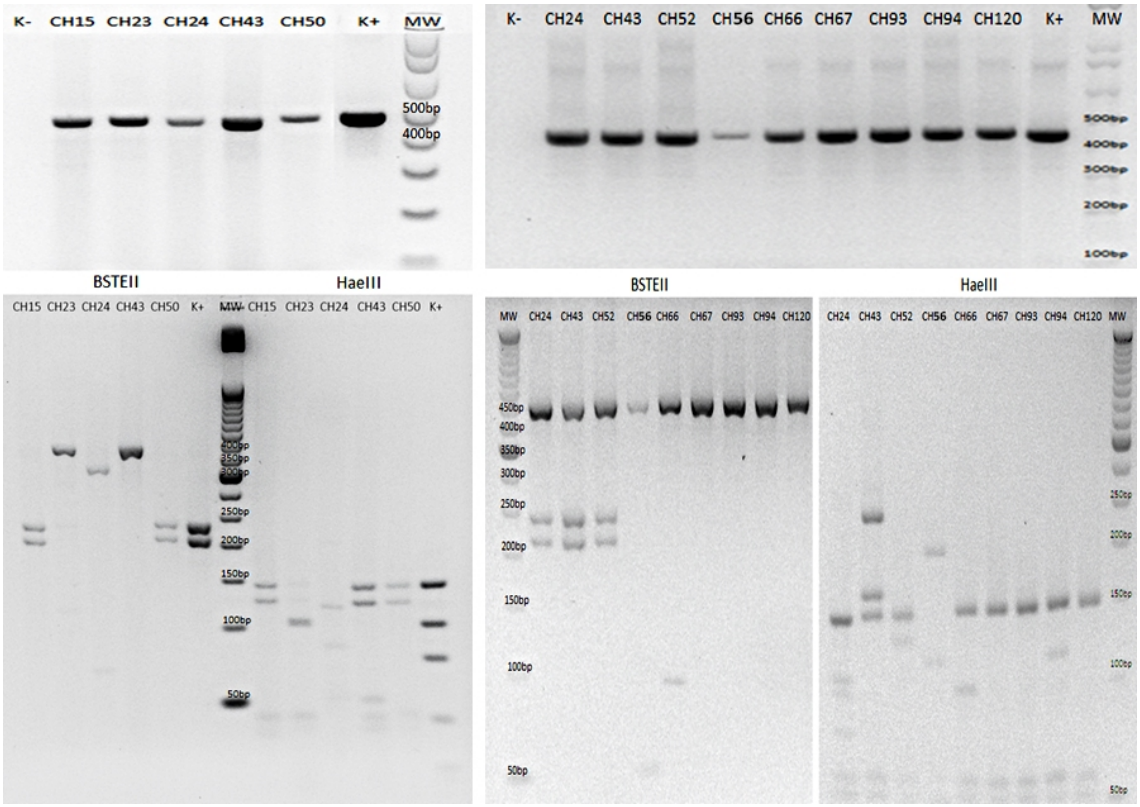


Fig. 3.5 Hsp65 amplicons (441 bp) and restriction patterns of the spleen samples from Calich (CH) obtained using the enzymes BSTEII and HaeIII corresponding to *Mycobacterium* spp. (MW = 1 Kb plus and 50 bp DNA Ladder; K+ = positive control).

Five of our sequences were deposited on the GenBank database, with the following accession number: KR779935, KX250267, KX250268, KX250269, KR779934, corresponding to STA11, STA15, STA33, STA40, and C27 respectively (Fig. 3.6).

Furthermore, the phylogenetic analysis revealed 6 sequences that clustered with hsp65 sequences of *M. marinum* confirming PCR-RFLP results, while the 4 sequences clustered with different strain of *Mycobacterium* spp., as shown in the original NJ tree (Fig. 3.7).

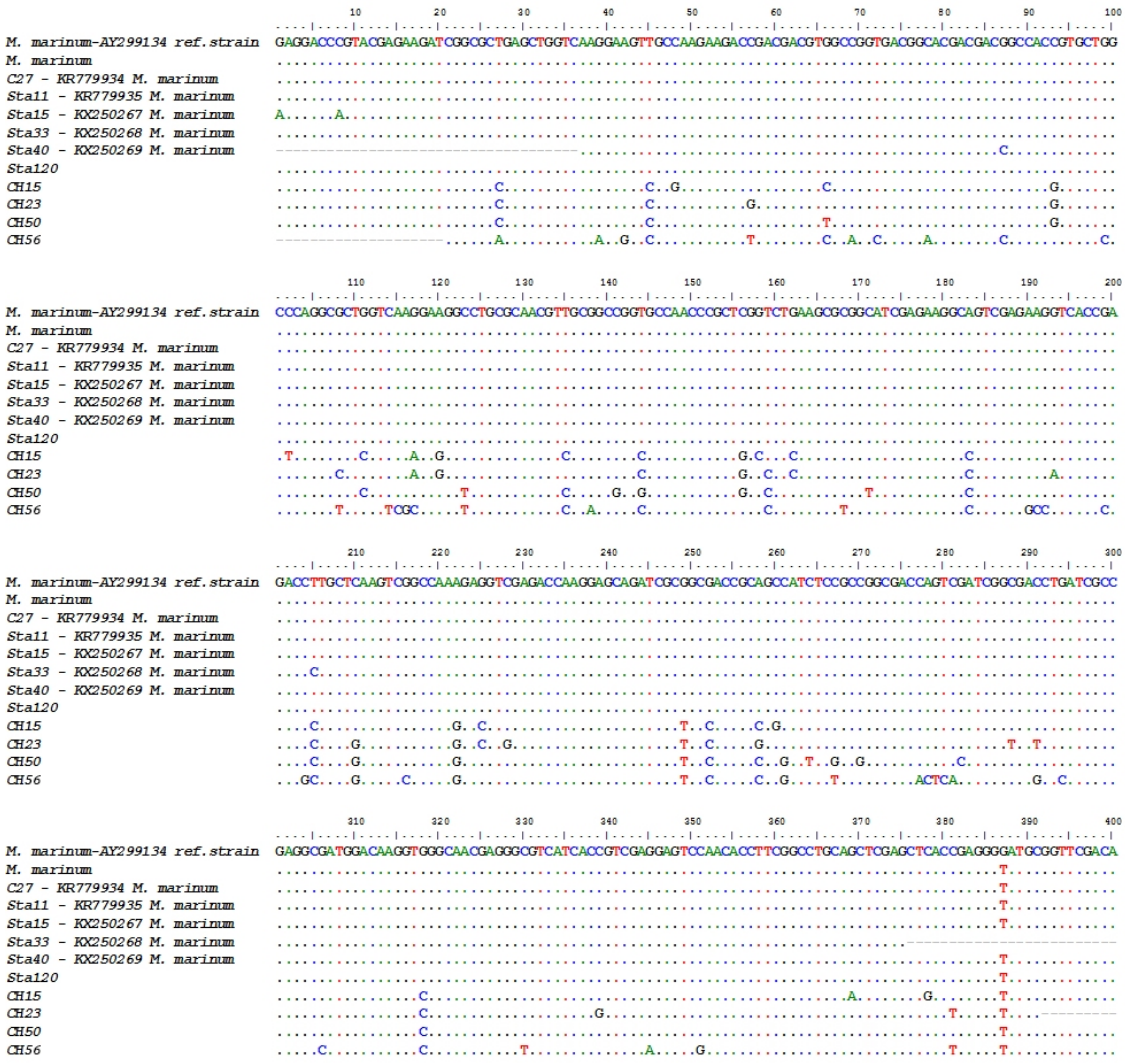


Fig. 3.6 Alignment of some sequences sharing>99% nucleotide identity with hsp65 sequences of *Mycobacterium marinum*.

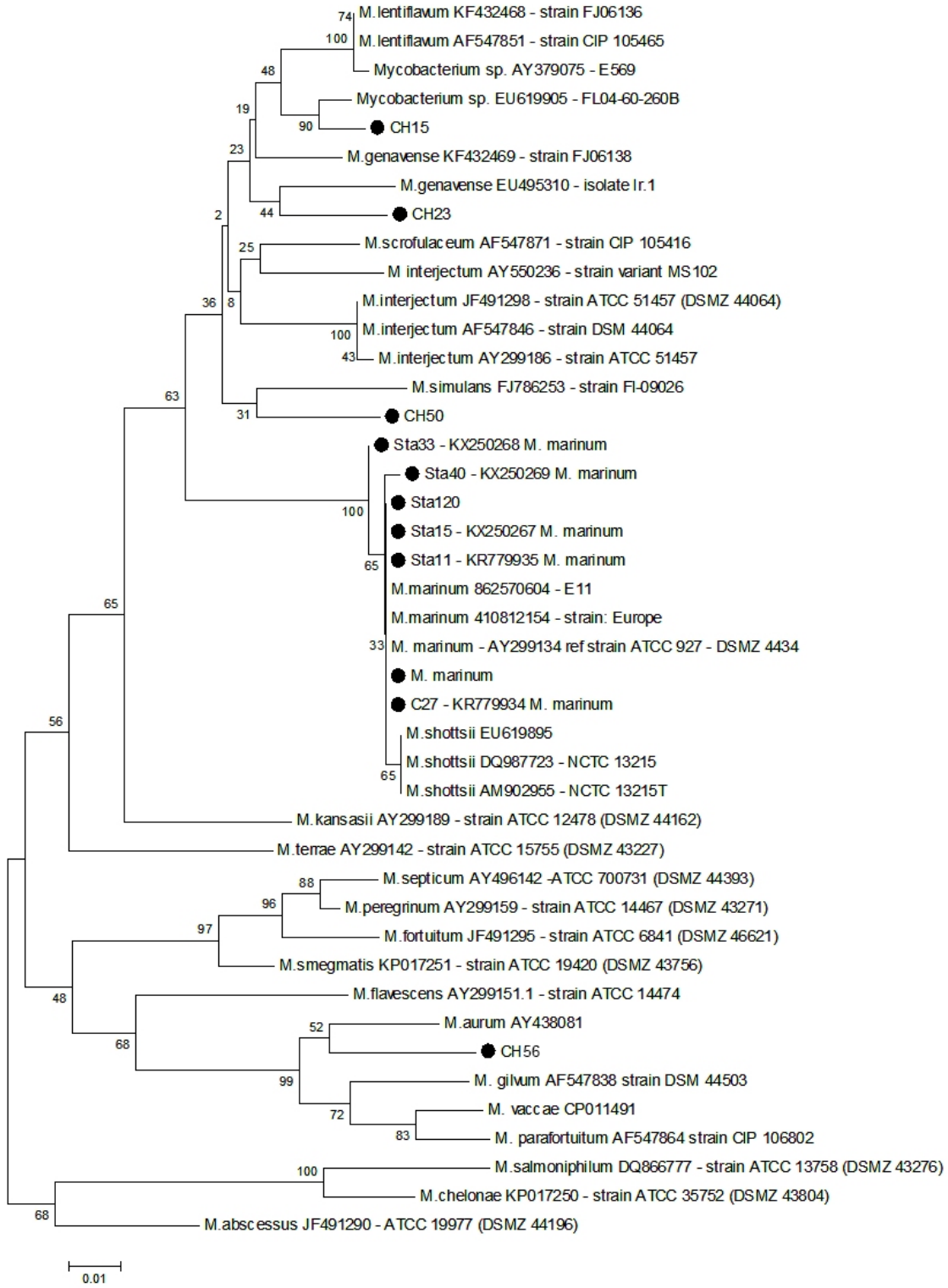


Fig. 3.7 Neighbor-Joining (NJ) tree generated from a 441bp sequence of hsp65 gene from 10 mullets spleen samples. Bootstrap values (expressed as percentages of 1000 replicates) are shown at branch points. The evolutionary distances were computed using the Kimura 2-parameter method. Phylogenetic analyses were conducted in MEGA.

3.4 Discussion

In this study, we examined for the first time the occurrence of mycobacteriosis in extensively reared mullets from 4 lagoons of the central-western Mediterranean Sea. In Cabras lagoon were found 2 positive samples out of 148 (1%), in San Teodoro lagoon the positivity was 11 subjects out of 138 fish sampled (8%) and in Calich lagoon was 12/120 (10%). Marceddi lagoon did not show any positive case.

Mycobacteriosis is quite difficult to detect even because clinical symptoms are absent in the acute forms and are nonspecific in the chronic ones (Kazda *et al.,* 2009). Than in the past, the incidence of mycobacteriosis has been increasing dramatically and the consequence of this disease in aquaculture is still poorly understood. Species-specific interactions, overcrowded conditions and environmental stressors could play a central role in mycobacteriosis epidemiology (Jacobs *et al.,* 2009). Moreover, mycobacteriosis in fish has not been properly investigated by the simultaneous application of histopathological, bacteriological and molecular biology methods (but see Pourahmad *et al.*, 2014; Timur *et al.*, 2015). Therefore, this disease is often underdiagnosed and information on its effects on farmed fish is rather limited (Bozzetta *et al.,* 2010).

In the last years, several studies have evidenced that the progression of mycobacterioisis is related to the different pathogenicity of the various mycobacterial species but also to the host fish species resistance (Decostere *et al.*, 2004 and references therein). Actually, host susceptibility in fish mycobacteria was evidenced in several experimental transmission studies. Some authors reported that striped bass is more susceptible to *Mycobacterium marinum* infection than hybrid tilapia (Wolf & Smith, 1999), while zebrafish is more susceptible than medaka (Broussard & Ennis, 2006) and hybrid striped bass (Ostland *et al.*, 2008).

The recent dramatic increase of culturing and molecular techniques led to a more precise identification and characterization of *Mycobacterium* species. These methods allowed to identify *M. marinum* as the major responsible of this disease in cultured fish. In fact, a number of infections caused by this species have been documented by several authors (Hedrick *et al.*, 1987; Diamant *et al.*, 2000; dos Santos *et al.*, 2002; Ostland *et al.*, 2008; Yanong *et al.*, 2010; Ackleh *et al.*, 2014) worldwide, but only a few have been reported from Italy (Salogni *et al.*, 2007, 2009; Bozzetta *et al.*, 2010; Mancuso, 2015).

In our report, the presence of visceral granulomas was histologically detected in 25 out of 495 mullets examined (5%). Except for a single specimen where granulomas were

detected exclusively in the heart, these lesions were mainly evidenced in spleen (92%) and in liver (28%).

Based on this evidence, spleen and liver can be considered as the two organs earlier affected as already reported by other authors (Jacobs *et al.*, 2009; Pourahmad *et al.*, 2014).

In details, six cases (5 from San Teodoro and 1 from Cabras) were detected positive to *M. marinum.* In all of these cases granulomas ZN positive were observed in spleens (6/6) and in liver (4/6). Cultural test performed revealed positivity for *Mycobacterium marinum* in 6 out of 6 spleen whereas only 1 out of 4 liver were positive. The molecular method identified *Mycobacterium marinum* in all 6 fish and definitive identification was based on sequencing. Summary of concordance in case of *Mycobacterium marinum* infections obtained by histology, cultural and phenotypic evaluation and sequence analysis of the hsp65 gene was 100%.

Four cases sampled from Calich lagoon were detected positive to *Mycobacterium* spp. In all of these, granulomas and acid fast bacilli were observed in all spleen (4/4). Cultural test performed revealed positivity for *Mycobacterium* spp. in 1 out of 4 spleen. Almost certainly the use of a decontamination during cultivation lowered the viability of mycobacteria as previously reported by Palomino *et al.* (1998). This fact could lead to underestimate mycobacterial infection in bacteriological analyses with respect to histological evidences. The molecular method as well as sequencing identified *Mycobacterium* spp. in all of the 4 fish examined (Table 3.1). Overall, *Mycobacterium* spp. identification by PCR-hsp65 and sequencing were significantly more accurate than cultural methods, showing 100% of positivity vs. 25%, respectively.

In addition fifteen specimen (8 from Calich; 6 from San Teodoro and 1 from Cabras) showed granulomas and acid fast bacilli positive to ZN in spleens (13/15), in liver (3/15) and in heart (1/15). Cultural test performed revealed positivity for *Mycobacterium* spp. in 5 out of 13 spleens whereas 3 out of 3 liver were positive. Among the 13 spleens, sampled PCR-hsp65 failed to identify restriction fragment fitting with atypical mycobacteria and failed DNA sequencing. Histological methods and culture identification for *Mycobacterium* spp. were in accordance with the 43% (6/14) of the examined cases.

Although PCR and culture tests detected at the same rates of mycobacteriosis caused by *M. marinum* in the mullets examined, histopathology showed a higher detection of this disease if compared to previous reports (Kaatari *et al.*, 2005; Whipps *et al.*, 2008).

Thus, histopathology remains the most common method for the first screening of fish mycobacteriosis, but culture and PCR-based methods are required for species identification. Furthermore, our study demonstrated that DNA sequencing of the PCR amplicons was essential both to demonstrate that *M. marinum* was the primary cause of mycobacteriosis in the mullets analyzed and to exclude the presence of mixed infections (Poort *et al.*, 2006). Among the 4 species investigated, *L. ramada* were the most affected ones by granulomas in visceral organs (11 specimens).

As far as the sampling site is concerned, however, mullets with ZN positive granulomas were mainly fished in the Calich and San Teodoro lagoons. This latter biotope is a heavily euthrophicated, coastal pond connected to the sea through a narrow mouth, which is often impounded by sand and *Posidonia oceanica* debris. A substantial impact on this environment is attributable to the discharge of municipal wastewaters from the town of San Teodoro. Moreover, the lagoon receives from Rio San Teodoro and Rio Filicani nutrient rich freshwater. Recently, massive algal blooms due to the pollution caused dramatic mortalities to the biota (Munari & Mistri, 2007), and the marketing of bivalves cultured in the lagoon was forbidden due to the critical conditions of the sanitary state of the water. Even in the Calich lagoon an inadequate purification of urban and agricultural wastewater represents the main source of contaminants. As a result, dystrophic crises, frequently associated with microalgal blooms, can occur during summer, leading to extensive fish mortality (Bazzoni *et al.*, 2013).

Other authors that investigated mycobacteriosis in mullets (Varello *et al.*, 2014) reported a higher prevalence of ZN positive granulomas (19.5%) than what we observed. This discrepancy could be related to the different environmental characteristics of the two sampling areas. Moreover, our results differ to those previously reported by other authors that identified *M. fortuitum* and *M. abscessus* as the two most frequent mycobacteria in mullets (Perez *et al.*, 2001; Varello *et al.*, 2014). Actually, we identified *M. marinum* by PCR and Restriction Enzyme Pattern Analysis and DNA sequencing, as the primary causative agents of mycobacteriosis in Mugilidae, as reported in literature for a wide range of saltwater species (Decostere *et al.*, 2004; Gauthier & Rhodes, 2009).

3.5 Conclusion

Based on our results, we evidenced for the first time the presence of mycobacteriosis in reared mullet affected from Sardinia (Central western Mediterranean). Histology

remains the most reliable tool for identification of diseased fish although culture, PCR-based methods and DNA sequencing are essential for species identification.

This finding is worthy of attention not only because it suggests the need for a higher monitoring effort to determine the welfare status of the farmed fish but, above all, for the potential zoonotic implications that this disease can have for fisheries and aquaculture operators. In Sardinia, in particular, the handling of mullets by fishermen is not only limited to fishing practices, but is very common for the preparation of a gourmet delicacy called "bottarga" (fish roe), which process method requires the handling of fish viscera (Murgia *et al.*, 2002).

N.B. Some contents of this chapter has been published in:
Antuofermo E., Pais A., Polinas M., Cubeddu T., Righetti M., Sanna M.A., Prearo M. (2016, in press). Mycobacteriosis caused by *Mycobacterium marinum* in reared mullets: first evidence from Sardinia (Italy). Journal of Fish Diseases. doi: 10.1111/jfd.12515.

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Chapter 4

FIRST EVIDENCE OF INTERSEX CONDITION IN EXTENSIVELY REARED MULLETS FROM SARDINIAN LAGOONS

4.1 Introduction

The family Mugilidae is one of the most ubiquitous in coastal waters of the world (Crosetti & Blaber, 2016). These fishes, generally known as mullets, are widely distributed in all temperate and tropical seas and live in several habitats, including river, estuarine and inland brackish waters. A number of species belonging to this teleost family are economically important for fisheries and aquacultural activities, as they are a major food source in several regions worldwide.

Mullets are cultured in semi-intensive and intensive systems in many areas of the world, but especially in extensive systems such as confined coastal lagoons in the Mediterranean basin. In this geographical area, wild fry of different euryhaline species [(i.e. *Chelon labrosus* (Risso, 1827), *Liza aurata* (Risso, 1810), *Liza ramada* (Risso, 1827), *Liza saliens* (Risso, 1810), and *Mugil cephalus* Linnaeus, 1758)] usually move in large schools from the sea into inland transitional waters, where they find more favourable trophic conditions (Crosetti & Blaber, 2016). They spend here most of the growing phase before migrating to sea to spawn in surface waters. Like other species, however, mullets tolerate polluted habitats and are sensitive to several contaminants, also to those which can cause intersex conditions in fish (Ortiz-Zarragoitia *et al.,* 2014; Tancioni *et al.,* 2015, 2016).

The term intersex describes alterations in gonadal development with the simultaneous presence of male and female reproductive stages in the same gonad of a gonochoristic species. This condition also indicates the occurrence of testicular oocytes, testicular follicles, testis-ova or ovotestes (Hecker *et al.,* 2006; Bahamonde *et al.,* 2013). In particular, the presence of oocytes in the testes of adult or sub-adults males (i.e. the testicular oocytes, TOs) represents the most commonly reported intersex condition in fish (Abdel-moneim *et al.,* 2015 and references therein). Histological examination can play a key role to evaluate gonadal alterations by detecting the presence of TOs (Stentiford *et al.,* 2003; Feist *et al.,* 2015). Furthermore, different levels of intersex condition in fish initially evidenced by several authors (Jobling *et al.,* 1998; Van Aerle *et al.,* 2001) were subsequently calculated using the ovotestis severity index (OSI), a ranking system developed by Bateman *et al.,* (2004).

Sexual disorders in fish have been principally attributed to many chemical contaminants, as the endocrine disrupting compounds (EDCs) which can produce effects similar to sex steroids (although EDCs can also influence additional mechanisms not directly estrogen dependent involved in the development of intersex condition in

fish; e.g. see Bahamonde *et al.,* 2013). The EDCs are a wide range of chemicals compounds that can affect, among others, the Hypothalamic-Pituitary-Gonad-Liver (HPGL) axis of fish (Hachfi *et al.,* 2012). These substances include both natural estrogens and several synthetic chemicals such as pesticides, polychlorinated biphenyls (PCBs), phthalates, and alkylphenols (Allen *et al.*, 1999 and references therein). In particular, the 17α -ethinylestradiol (EE2) is a strong endocrine disruptor which mimics the effects of endogenous 17-β-estradiol (E2) (Blewett *et al.*, 2014). It has been observed that EE2 can cause altered oogenesis in females and intersex in males, with production of vitellogenin (the female specific yolk protein precursor in oviparous species) and early-stage eggs in their testes (Tyler *et al.*, 1998; Kidd *et al.*, 2007).

Fish are among the most studied organisms for the effects of chemical contaminants on the development and reproductive processes. A high rate of intersex conditions and other gonadal disorders have been actually detected in many freshwater and marine species from habitats exposed to domestic and industrial wastewaters (Abdel-moneim *et al.,* 2015). In coastal and estuarine environments, in particular, euryhaline fish living in polluted waters like Mugilidae frequently show sexual alterations and are considered as sentinels of exposure to EDCs (Ortiz-Zarragoitia *et al.*, 2014).

In the Mediterranean Sea, extensive mullet culture is still based on the collection of wild fry born in offshore marine waters (as their hatchery production is still not practiced at a commercial level) which return to coastal brackish waters within few months after hatching.

The aim of the present work was to evaluate for the first time the occurrence of intersex condition in cultured Mugilidae from Sardinia (Italy), an insular region in which the extensive farming of these eurhialine fishes is a traditional activity (Cataudella *et al.*, 2015). Consequently, adult specimens of several mullet species were sampled in two different lagoons devoted to extensive aquacultural practices, in order to identify putative alterations in gonads and in gamete development.

4.2 Material and methods

4.2.1 Study area

In this study, two brackish habitats of Sardinia island devoted to extensive aquaculture were examined: (a) the Marceddì lagoon (central-western Sardinia, Fig. 4.1) which is part of the Oristano Lagoon Gulf-system, a wetland area traditionally devoted to fishing and aquacultural practices (Cataudella *et al.*, 2015); and (b) the San Teodoro lagoon (north-eastern Sardinia, Fig. 4.1) in which extensive fish farming have been developed since the 1980s.

Fig. 4.1 Study area and sampling locations.

4.2.2 Sampling

One hundred and fifty-eight adult fish belonging to the family Mugilidae were sampled respectively at Marceddì (8 *Chelon labrosus*, 48 *Liza aurata*, and 4 *Mugil cephalus*) and San Teodoro (24 *C. labrosus*, 53 *L. aurata*, and 21 *M. cephalus*) lagoons in late summer 2014. Sampling techniques are reported in detail in Chapter 2.

4.2.3 Histology

Samples taken from anterior, middle and posterior part of both right and left fixed gonads of each specimen were dehydrated in graded alcohol, cleared with xylene and

paraffin embedded. Three sections (approximately taken at one third of each portion) were cut at 3-micron-thickness, stained with hematoxylin-eosin and subsequently observed at light microscopy (Nikon Eclipse 80i).

Gonadal maturation stages were assessed using the classification described by McDonough (2005). Oocyte development was classified into five distinct reproductive stages: immature stage (F1), with previtellogenic oocytes (<80 µm), no evidence of atresia and ovary wall is very thin; developing stage (F2), where oocytes are greater than 120 µm and vitellogenesis occurs after they reach 180 µm in size, showing yolk globules and reaching more than 600 µm; running stage (F3), where vitellogenesis is completed and the whole cytoplasm is filled with yolk granules; atretic stage (F4), in which oocytes undergone degeneration; and inactive stage (F5), where there are previtellogenic oocytes with only traces of atresia.

Furthermore, spermatogenetic stages were classified as follows: immature stage (M1), with spermatogonia and little or no spermatocytic development; developing stage (M2), with predominance of primary and secondary spermatocytes; running stage (M3), with predominance of spermatozoa; spent stage (M4), in which no spermatogenesis occurs with some residual spermatozoa; and inactive stage (M5), with little or no spermatocytic development and empty lobules. If different development stages were observed in the same gonad, the sexual maturity of each mullet was classified in relation to the most advanced stage of maturation found.

When a gonadal disorder was observed, the intersex condition was evaluated following Hecker *et al.* (2006). In particular, if single or clustered oocytes were present in testicular tissue the condition was termed testis-ova (TOs). For each gonad section, photomicrographs were acquired with a Nikon Digital Sight DS-U1 camera, and number, development stage (previtellogenic, cortical alveolar, vitellogenic) and distribution of oocytes (focal, diffuse, cluster, zonal) throughout testis were evaluated. This was done to calculate the so-called ovotestis severity index (OSI), a ranking system for assigning a severity rating to each intersex fish:

$$\mathrm{OSI} = \left[\frac{\sum(D_1 \times D_2)}{X}\right]$$

where D_1 is the most advanced development stage of oocytes within a field of view (score 1-5), D_2 is the distribution of oocytes within a field of view (score 1-4), and X is

the total number of fields of view examined (Bateman *et al.*, 2004 and references therein).

Ten fields of each tissue section were examined at 10× magnification. In detail, the development stages of oocytes were scored as follows: stage 1 (oogonia), stage 2 (oocytes in early perinucleolus stage), stage 3 (oocytes in late perinucleolus stage), stage 4 (oocytes in cortical stage) and stage 5 (oocytes in mature vitellogenic stage). The distribution of oocytes were instead scored as follows: stage 1 or focal distribution (when a single oocyte was present within a field of view), stage 2 or diffuse distribution (when a number of distinct oocytes were observed in a field), stage 3 or cluster distribution (when more than one but less than five associated oocytes were present in a field), and stage 4 or zonal distribution (when multiple closely groups of oocytes were seen within a field).

4.3 Results

Different stages of sexual development were observed in the three mullet species from the two lagoons (Table 4.1, Figs. 4.2 & 4.3). When examined macroscopically, immature female gonads were pinkish and translucent, with a circular cross section of about 0.5 cm. Mature female gonads, instead, had a yellowish-orange color, with a round cross section from 1 to 2.5 cm. Immature male gonads appeared filiform, pinkish and translucent with a cross section of about 1-2 mm, whereas mature male gonads had a whitish-milky aspect, with a triangular cross section of about 1-1.5 cm (Fig. 4.2). Microscopically, gonads were classified as follows: six females (all at F1 stage) and one male (M2) of *Chelon labrosus*, 42 females (13 at F1, five at F2 and 24 at F3) and four males (one at M2, three at M3) of *Liza aurata*, one female (F1) and two males (one at M1 and one at M2) of *Mugil cephalus* were found in the Marceddì lagoon. On the other hand, 12 females (11 at F1 and one at F2) and seven (all at M1) males of *C. labrosus*, 35 females (23 at F1, two at F2 and 10 at F3) and 15 males (two at M1, six at M2 and seven at M3) of *L. aurata*, eight females (two at F1, four at F2 and two at F3) and 11 males (one at M1, one at M2 and nine at M3) of *M. cephalus* were found in the San Teodoro lagoon. Only a *L. aurata* female specimen sampled at this latter lagoon was not microscopically evaluated. The other mullets examined (13 out of 158; 8.2% of the total) were affected by an intersex condition of the gonads [12 classified as testis-ova (TOs) and one as mixed gonadal tissue (MGT) following Hecker et al. (2006)]. In detail, four subjects with gonadal disorders [(one *C. labrosus* (MGT, Fig. 4.4), two *L. aurata* and one *M. cephalus*)] were found in the Marceddì lagoon and the other nine (five *C. labrosus*, two *L. aurata* and two *M. cephalus*) in the San Teodoro lagoon (Table 4.2). Macroscopically, almost all of these fish had gonads showing an aspect similar to those of immature males. The only exception was represented by two *M. cephalus* specimens (sampled at Marceddì and San Teodoro, respectively), which gonads were comparable in colour and form to those of mature males (Fig. 4.2).

Microscopically, the male component of the 12 TOs observed was classified as follows: one at M1 and one at M2 stage *L. aurata*, and one at M3 *M. cephalus* at the Marceddì lagoon; one at M1 and one at M3 stage *L. aurata*, four at M1 and one at M2 *C. labrosus*, and two at M3 *M. cephalus* were instead found at the San Teodoro lagoon (Fig. 4.3).

It is also worth mentioning that the *C. labrosus* specimen sampled at the Marceddì lagoon (MR37, Table 4.2) showing a monolateral mixed gonadal tissue (MGT) had one

gonad with a normal ovary tissue and the other with several primary oocytes within a testicular tissue (Fig. 4.4). As far as ovotestis severity index (OSI) is concerned, it varied from a minimum of 0.1 to a maximum of 2.4. Thus, all the recorded values were included in the stage 1 (i.e. OSI score = 0÷5) of severity categories proposed by Bateman *et al.* (2004).

Table 4.1 Different gonadal stages of mullets sampled in the two lagoons.

		Females			Males			Intersex	
Site	Species	F1	F2	F3	M1	M2	M3	TOs	MGT
Marceddì	*Chelon labrosus*	6	-	-	-	1	-	-	1
	Liza aurata	13	5	24	-	1	3	2	-
	Mugil cephalus	1	-	-	1	1	-	1	-
San Teodoro	*Chelon labrosus*	11	1	-	7	-	-	5	-
	Liza aurata	23	2	10	2	6	7	2	-
	Mugil cephalus	2	4	2	1	1	9	2	-

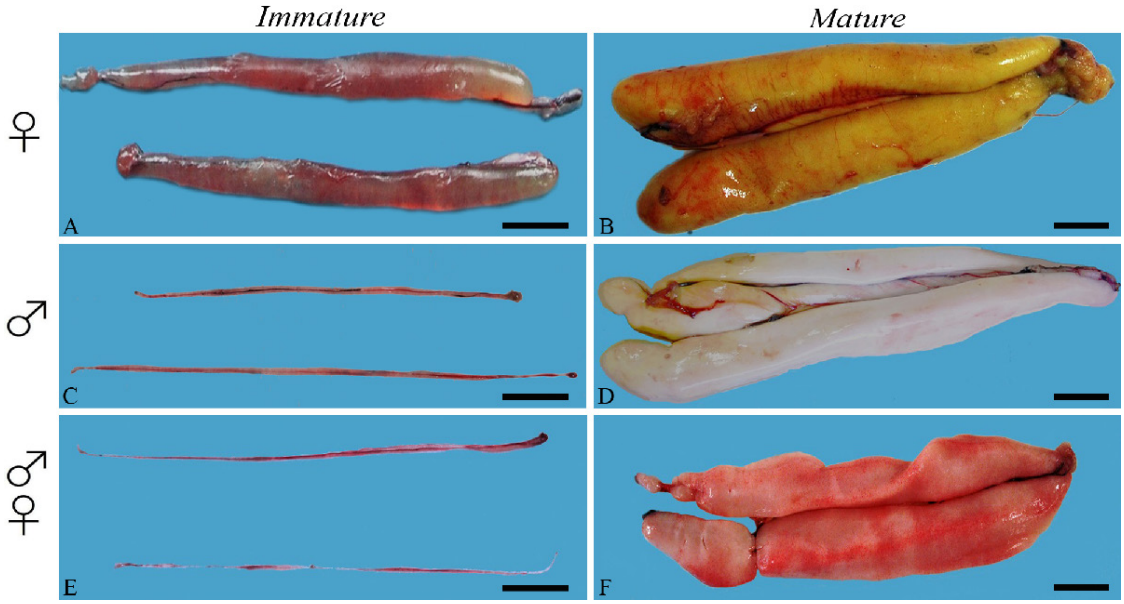

Fig. 4.2 *Mugil cephalus*. Gross structure of immature and mature females (A, B), males (C, D) and intersex (E, F) gonads (bar=10 mm).

Table 4.2 Biometric features and Intersex Index of the mullets affected by intersex condition (MRn=Marceddì; STAn=San Teodoro).

Case	ID	Species	Total length (cm)	Total weigth (g)	Gonad weigth (g)	Intersex Index
1	MR37	*Chelon labrosus*	40.4	755.3	1.2	-
2	STA48	*Chelon labrosus*	25.1	162.9	nd	0.6
3	STA62	*Chelon labrosus*	28.6	222.7	0.1	2.4
4	STA65	*Chelon labrosus*	29.8	268.4	0.2	1.8
5	STA66	*Chelon labrosus*	30.9	271.4	0.1	0.8
6	STA86	*Chelon labrosus*	34.2	401.2	0.2	0.1
7	MR68	*Liza aurata*	27.7	224.0	0.1	2.0
8	MR78	*Liza aurata*	26.3	174.7	0.2	0.1
9	STA3	*Liza aurata*	28.7	196.2	0.1	0.6
10	STA9	*Liza aurata*	24.2	109.0	0.1	0.7
11	MR89	*Mugil cephalus*	38.7	571.6	1.3	0.2
12	STA114	*Mugil cephalus*	46.2	1101.2	5.7	0.5
13	STA117	*Mugil cephalus*	41.6	812.0	0.4	0.1

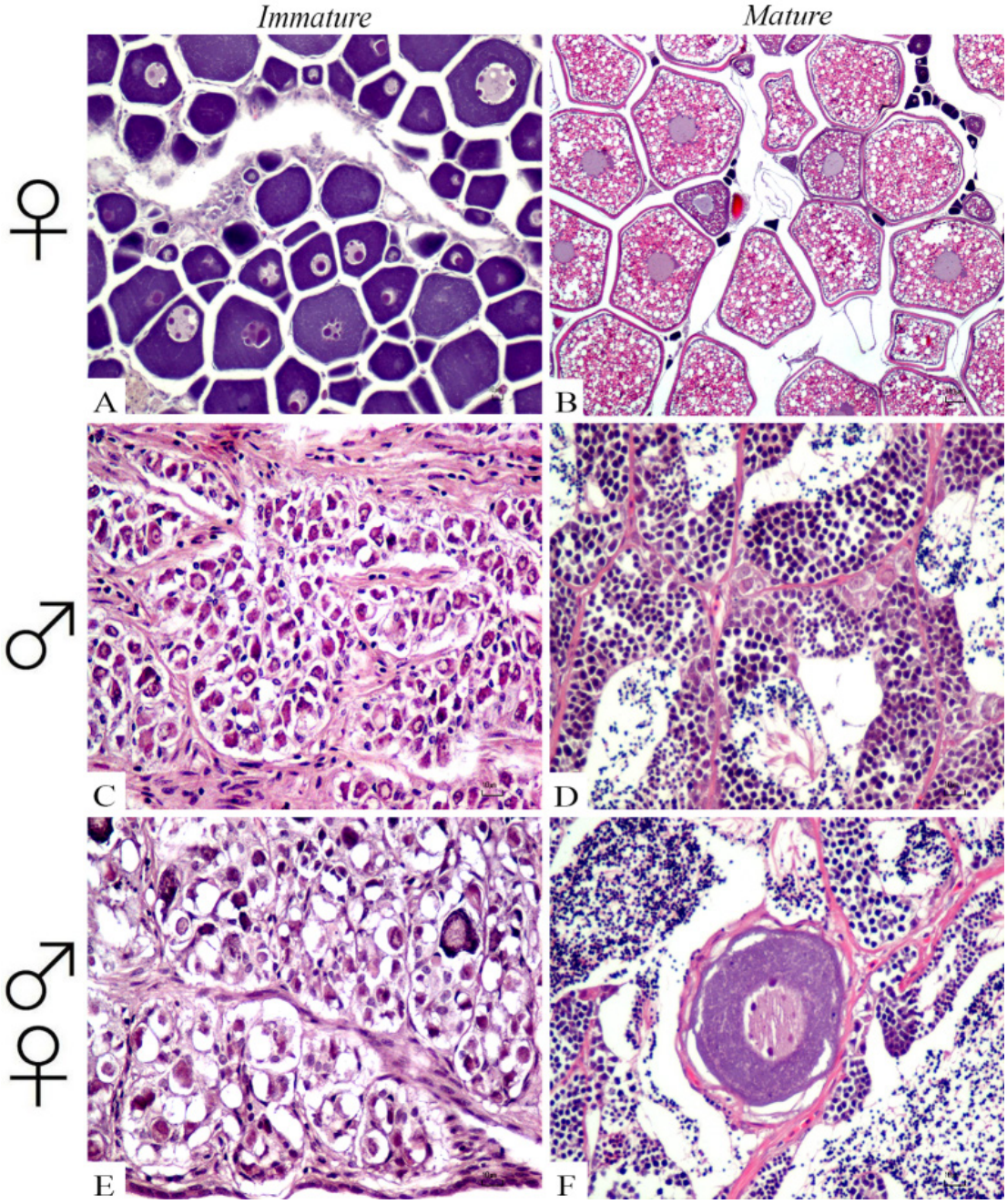

Fig. 4.3 *Mugil cephalus*. Microscopical features of: immature and mature female gonads (A = previtellogenic oocytes, HE 20×; B = vitellogenic oocytes, HE 10×); immature and mature male gonads (C = seminiferous lobules with high prevalence of spermatogonia, HE 40×; D = seminiferous lobules with different maturation stages of testis germ cells, HE 40×); and TO gonads (E = previtellogenic oocyte at chromatin nucleolus stage within immature testicular tissue, HE 40×; F = previtellogenic oocyte at cortical alveoli stage within mature testicular tissue, HE 40×).

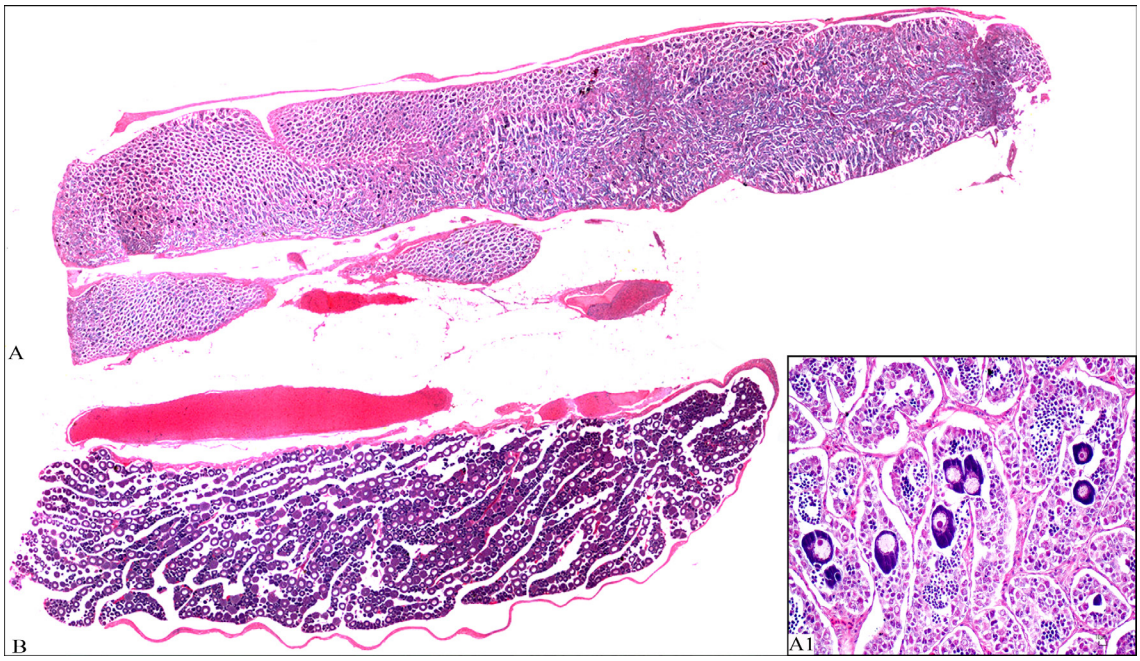

Fig. 4.4 *Chelon labrosus*. Monolateral mixed gonadal tissue (MGT): A = testis with previtellogenic oocytes within the seminiferous lobules (A1, HE 40×); B = immature female gonad with previtellogenic oocytes (HE 2×).

4.4 Discussion

In this study, we reported for the first time the occurrence of intersex condition in extensively reared mullets from two lagoons of the central-western Mediterranean Sea. In the Marceddì lagoon we found intersex gonads in four fish out of 60 sampled (6.7% of the total; 12.5% in *Chelon labrosus*, 4.2% in *Liza aurata*, and 25% in *Mugil cephalus*, respectively), while in the San Teodoro lagoon this gonadal disorder was observed in nine of 98 subjects examined (9.2% of the total; 20.8% in *C. labrosus*, 3.8% in *L. aurata*, and 9.5% in *M. cephalus*, respectively).

In the aquatic habitat there are numerous contaminants (the so-called endocrine disruptor compounds, EDCs) that are able to interfere with the endocrine system with damaging effects on growth, behavior, reproductive and immune system of aquatic organisms. Morphological modification and abnormal development of the gonads, including intersex, have been observed in a number of fish species living in polluted waters (Jobling *et al.,* 1998; Puzzi *et al.,* 2005), and have been linked to exposure to EDCs (Scholz & Klüver, 2009) and other chemicals released with human and industrial discharges (Van Aerle *et al.,* 2001; Tetreault *et al.,* 2011). In particular, gonadal disorders have been reported worldwide for several mullet species: *Chelon labrosus* was studied by Puy-Azurmendi *et al.* (2013) and Bizzarro *et al.* (2014) at the Bay of Biscay (Spain); *Liza ramada* by Bayhan & Acarli (2006) at the Homa Lagoon (Turkey) and Tancioni *et al.* (2015, 2016) at the Tiber estuary (Italy); and *Mugil cephalus* by Ferreira *et al.* (2004) at the Douro estuary (Portugal) and Aoki *et al.* (2010) in the Korean and Japanese coastal waters.

To the best of our knowledge, this is the first report of cases of gonadal disorder in *Liza aurata* (2 specimens at Marceddì and 2 at the San Teodoro lagoon) which highlights the susceptibility to contaminants also for this species. Thus, as different mullet species can inhabit the same coastal brackish environment [in particular the lagoons of the Mediterranean basin, Cataudella *et al.* (2015)], because they are able to use the *pabulum* by directly grazing the bottom mud or using plant-detritus (Crosetti & Blaber, 2016), the outcomes of our work evidenced the simultaneous presence of gonadal abnormalities (TOs) in several mullet species cohabiting in two different aquatic biotopes. In particular, it is important to note that the Marceddì lagoon is located near an area of intensive agricultural and zootechnical activities which wastewaters can be a potential source of chemicals that contribute to intersex formation in fish. Until the early 1990s, mining was also present in the drainage basin of the Marceddì lagoon

where high concentrations of heavy metals [which can also contribute to intersex formation; see e.g. Hinck *et al.* (2007)] have been found in sediments (Magni *et al.*, 2006). The San Teodoro lagoon, instead, receives municipal wastewaters from the small town of San Teodoro (one of the most important tourist centre in north-eastern Sardinia), and nutrient rich freshwater from two little rivers which sometimes discharge into it untreated wastewaters from the surrounding area (Antuofermo *et al.*, 2016).

In our results, TOs (12 of the 13 gonadal abnormalities observed) were the most represented intersex condition in mullets, as previously reported by several authors for fish and amphibians (Abdel-moneim *et al.*, 2015 and references therein). Only one specimen (*C. labrosus* from Marceddì) showed a monolateral mixed gonadal tissue (MGT) which may be considered as an example of rudimentary hermaphroditism (because male and female gonads were present in the same fish; Hecker *et al.*, 2006), whose origin could be related to natural or exogenous factors.

In fish, testes have to be examined histologically to detect cases of intersex because the testicular tissues can often appear normal at gross examination. In mullets, a high rate of intersex gonads (23.1% of the fish examined) was found by Tancioni *et al.* (2016) in a wild population of thinlip grey mullet *L. ramada* from a polluted estuary in central Italy. Similar rates of intersex gonads were observed in other mullets sampled from other polluted environments: 21% in flathead grey mullet (*M. cephalus*) from the Douro estuary in northern Portugal by Ferreira *et al.* (2004), and up to 50% in thicklip grey mullet (*C. labrosus*) from the Bay of Biscay in northern Spain (Puy-Azurmendi *et al.*, 2013; Bizarro *et al.*, 2014).

Several studies have evidenced that intersexuality can vary from mild to severe stages according to the number, maturity, and distribution of oocytes within the normal testicular tissue (Bateman *et al.*, 2004 and references therein). For this reason, we decided to attribute a score to each intersex specimen according to the ovotestis severity index (OSI) proposed by Bateman *et al.* (2004). This index ranges from a stage 1 indicating mild intersex (based on the presence of few previtellogenic oocytes within a normal testis) to a stage 3 showing a severe abnormality of the testicular tissue, which is replaced by numerous oocytes in advanced stage of development. All TOs found in our study were classified as stage 1 of OSI (intersex individuals exhibited a mild grade of intersex condition corresponding to testis with scarce previtellogenic oocytes) (Jobling *et al.,* 1998; Ferreira *et al.,* 2004). The application of OSI was not suitable only in one case of intersex in which we observed a mixed gonadal tissue (*sensu* Hecker *et al.*,

2006), as suggested by Bateman *et al.* (2004).

Only few recent studies have reported the severity of the intersex condition in mullets. For example, according to the classification proposed by Jobling *et al.* (1998), Bizzaro *et al.* (2014) and Sardi *et al.* (2015) observed mild to moderate severity values in *C. labrosus* from the Basque coast (Spain). Several authors reported that mild severities of gonadal intersex (i.e. stage 1 of OSI) are generally not associated with impairments in the reproductive activity of fish (Abdel-moneim *et al.*, 2015 and references therein), although adverse reproductive effects are likely when severity of intersex condition increases (Jobling *et al.*, 1998, 2002; Harris *et al.*, 2011). At this regard, a direct correlation between the incidence and the severity of sexual disruption in fish, and natural or synthetic chemicals (EDCs) in the waters was already evidenced by Jobling *et al.* (2006). In view of this, the timing of exposure to these compounds can be critical as fish seem to be most susceptible to EDCs just after hatching or as juveniles before sex differentiation (Jobling *et al.*, 1998; Bateman *et al.*, 2004). In particular, there is a sensitivity period occurring during the first few months of larval development (Devlin *et al.*, 2002) in which a transitory exposure to xenobiotics can feminize male fish (Ortiz-Zarragoitia *et al.*, 2014). This period is usually spent offshore by the fry of mullets, and the consequent sexual differentiation (corresponding to juvenile recruitment into estuarine waters) can be affected by high concentrations of EDCs (Ortiz-Zarragoitia *et al.*, 2014).

However, a low level of gonadal intersex (<5%) may naturally occur in a number of gonochoristic fish species (Blazer *et al.*, 2007 and references therein). Although a relatively high percentage of intersex was observed at Marceddì and San Teodoro lagoons (6.7 and 9.2%, respectively), we cannot completely exclude a natural phenomenon (not related to EDCs exposure) due to innate physiological drivers giving rise for generally low grade intersex condition. In fact, even if Tancioni *et al*. (2015) affirmed that the prevalence of natural hermaphroditism in mullets is non-existent or very low, some cases were previously reported for *M. cephalus* (Franks *et al.*, 1998) and *L. ramada* (Bayhan & Acarli, 2006). Thus, it is also possible that there is a general lack of information on this specific topic for migratory fish like mullets, conversely to other more studied species (Bahamonde *et al.*, 2013).

In any case, the incidence of intersex condition in mullets can vary with the season. In fact, a seasonal pattern was evidenced in wild *L. ramada* by Tancioni *et al.* (2015) with high values recorded during the spawning and gonad development periods.

Analogously, Ferreira *et al.* (2004) and Aoki *et al.* (2010) observed the same phenomenon in *M. cephalus*. For this reason, to improve the assessment of the impact of environmental pollution on the reproductive status of wild fish, it would be better sampling two or to three weeks before their spawning season (Barrett & Munkittrick, 2010). Unfortunately, in this part of the study we considered only one sampling period (late summer 2014), when the availability of different species of extensive reared mullets in the capture chambers of fixed traps (lavorieri; Cataudella *et al.*, 2015) placed in the two lagoons mouths was maximum.

The percentage of intersex and the values of intersex severity index we found in mullets at both Marceddì and San Teodoro lagoons were lower in comparison with the previous cited works seems to drive our results to naturally intersex conditions. Nevertheless, Mugilidae can be considered as sentinel species in coastal biomonitor investigations (Waltham *et al.*, 2013) and in particular of exposure to EDCs in coastal and estuarine polluted environments (Ortiz-Zarragoitia *et al.*, 2014 and references therein). The study of their gonadal alterations should be further developed to evaluate anthropogenic threats (especially those linked to urban and industrial activities) to species of interest in aquaculture (Tancioni *et al.*, 2016).

4.5 Conclusion

Due to their economic importance, several problems linked to the extensive rearing of mullets have been studied in Sardinia in the last few years (Merella & Garippa, 2001; Murgia *et al.*, 2002; Antuofermo *et al.*, 2016). In fact, in this island, mullets are cultured not only for direct human consumption, but also for the preparation of a gourmet delicacy called “bottarga” (fish roe, in particular of *Mugil cephalus* and *Chelon labrosus*). Our findings suggest that a suitable management of this resource in extensive aquaculture activities have to take into account fish gonadal disorders. In fact, these sexual abnormalities may be underestimated also in other extensive reared fish species, particularly in coastal brackish environments polluted by intensive agriculture and animal husbandry practices.

N.B. The content of this chapter has been published in:
Antuofermo E., Ariu R., Burrai G.P., Polinas M., Sanna M.A., Esposito G., Prearo M. Pais A., (2016, in press). First evidence of intersex condition in extensively reared mullets from Sardinian lagoons (central-western Mediterranean, Italy). Italian Journal of Animal Science. doi: 10.1080/1828051X.2016.1264260.

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Acknowledgments

Happiness doesn't exist on the far side of distant mountains. It is within you, yourself. Not you, however, sitting in idle passivity. It is to be found in the vibrant dynamism of your own life as you struggle to challenge and overcome one obstacle after another, as you clamber up a perilous ridge in pursuit of that which lies beyond (Daisaku Ikeda, SGI President).

The words of my mentor Sensei guided me in this time of hard work helping me to never give up the goal even in the most difficult time. I wish to thank all those who contributed directly or indirectly to the victory of this great challenge.

First and foremost I want to thank my advisor Antonio Pais. It has been an honor to be his first veterinarian taking a PhD in the Department of Agriculture. I am also thankful to Marino Prearo for his fundamental contribution to this research work and for his helpfulness in the difficulties.

I am especially grateful to the members of all the research groups of the Department of Veterinary Medicine of the University of Sassari (Roberta Ariu, Giovanni Pietro Burrai, Tiziana Cubeddu, Giuseppe Esposito, Marta Polinas, Marina Antonella Sanna, Veronica Vitiello) and the "Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta" (Paola Arsieni, Marzia Righetti, Tommaso Scanzio) that have contributed immensely to this work and without their professional time this result would not have been achieved. I am especially grateful for the fun I had from my research group who helped me to smile in panic. I would also like to express my thankfulness to all the fishermen cooperative (San Teodoro, Calich, Cabras and Marceddì) that provided the mullets for the experimental work. I want to thank also the "Unità Operativa di Anatomia Patologica-AOU" of the University of Sassari (Vincenzo Marras, Rosanna Satta, Giulia Satta) to provide the cases related to tank granuloma in humans.

Last but not least, my deepest gratitude is for my mom for all her encouragement and especially for Mickey Mouse for teaching me to love.

Thanks