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NEW ADVANCES IN MOLECULAR EPIDEMIOLOGY OF EMERGING ENTERIC PARASITES IN FARMED GILTHEAD SEA BREAM (*SPARUS AURATA*) AND INSIGHTS INTO ZOONOTIC PARASITES OF NEW RELEVANCE IN ITALIAN FRESHWATER FISH

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ABSTRACT

Fish products are essential to the food and economic security for almost a billion people around the world. Parasitic diseases outbreaks are a major concern for aquaculture and fishery industries, and they may represent a major constraint for sectors' expansion. Fish-borne parasitic zoonoses due to the consumption of fishery products are an increasing phenomenon in European Countries.

This work maps the available information on *Enterospora nucleophila* and *Cryptosporidium molnari* presence in gilthead sea bream Mediterranean aquaculture. The existing diagnostic techniques for the detection of *E. nucleophila* and *C. molnari* were reviewed and a new *in situ* hybridization assay for the detection of *E. nucleophila* developmental stages is provided. Data from a survey involving 17 gilthead sea bream facilities showed a wide distribution pattern of *E. nucleophila* and *C. molnari*, with their detection in 41% and 47% farms, respectively. Data also showed a non-homogeneous distribution within different production phases and type of facilities. Parasites were mainly detected in post-weaning production phases, with higher detection of *E. nucleophila* at ongrowing in-land and cage-based rearing systems, and higher positivity percentages of *C. molnari* at pre-growing and in-land tanks. The absence of positivity at early production stages including eggs led to consider vertical transmission unlikely and to confirm the role of water in transmitting the infection.

This work aimed at shedding light on emerging zoonotic parasites in European freshwater fishery products. This work provided consistent information on *Eustrongylides excisus* epidemiology in Central Italy lakes and confirmed the presence of infective stages in freshwater fish. A molecular assay for the identification of adult and larval stages of *E. excisus* is reported.

A novel multiplex PCR assay for the simultaneous identification of *Opisthorchis felineus*, *Pseudamphistomum truncatum*, *Metorchis* spp., *Metagonimus* spp. and *Apophallus* spp., the main zoonotic or potentially zoonotic trematodes described in Europe, is also provided.

1. Introduction

Mediterranean marine finfish aquaculture is characterized by different systems and technologies. These include industrial hatcheries, land-based extensive and intensive grow-out systems up to sea cages, and mainly focus on producing European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*). Greece, Spain, Italy, Croatia and France are the leading production countries for these species on the European coasts of the Mediterranean basin.

These countries also produce hundred million juveniles of both species, which are traded in all the Mediterranean area. This volume of production leads to the usage of 600 million bacterial and viral vaccine doses every year. However, no vaccine is available for parasitic diseases and very few treatments are licensed and even less are proven to be effective (Fioravanti *et al*., 2020a).

The aetiology of parasitic diseases is frequently complex and involves, in addition to the parasite itself, several environmental and host-related cofactors, and human safety regulations.

This often makes treatments inconclusive (Fioravanti *et al*., 2020a). Therefore, control of parasitic diseases in aquaculture requires a holistic approach which encompasses all involved factors, based on a comprehensive understanding of the life cycle and transmission routes of parasites, and of the abiotic and biotic factors that can alter host-parasite interactions.

Parasitic diseases outbreaks are a major concern in aquaculture production. They are one of the reasons for trade barriers of aquaculture products and live fish, and a major constraint for aquaculture expansion (Tavornpanich *et al*., 2020).

Moreover, parasitic diseases cause a decrease in productivity, related either to an increase in mortality and to a decrease in growth. Parasites presence in culture systems may also induce a decrease in product economic value or they can constitute a threaten for human health, all contributing to a decrease in purchases and resulting in losses of billions of dollars per year (Tavornpanich *et al*., 2020).

Several factors related to both globalization and climate change are making parasitic diseases a major Public Health issue worldwide. The increase in water temperatures is driving major changes in freshwater, brackish and marine environments, whose overall impact has yet to be assessed and understood (Marcogliese, 2008; Lõhmus, and Björklund, 2015).

Thus, climate change together with globalization, alterations in legislation, population growth and movement, including urbanization, cultural changes, and many other factors may all result in specific pathogens, including fish-borne parasites, emerging, or reemerging, in unexpected ways (Robertson, 2018).

In European countries fish-borne parasitic zoonoses due to local and imported fish or fish products consumption is a recent, but increasing, phenomenon associated with increased consumption of fish per se, expansion of the aquaculture industry, new trends

in fish consumption, development of the cold chain to transport fish around the globe with insufficient inspection (Robertson *et al.*, 2014), and with fraudulent fish substitution (Williams *et al.*, 2020).

The emergence and re-emergence of parasitic diseases provide urgency to develop our understanding of the role of parasites in both wild and cultured fish populations and their potential to affect aquatic animals' and human health.

The understanding of parasites' epidemiology within affected host populations and the assessment of parasitic diseases impact over aquaculture and fishery productions deeply relies on the development of effective diagnostic tools and on their employment in surveillance programs.

For these reasons, this work purpose was to contribute to shed light on emerging enteric parasites of cultured gilthead sea bream and emerging zoonotic parasites in EU countries.

As concerning parasites presence in cultured fish population across the Mediterranean area, the research activities carried out during the Doctoral program mainly aimed at mapping the knowledge about *Enterospora nucleophila* and *Cryptosporidium molnari* occurrence in gilthead sea bream aquaculture.

Thus, the first purposes of this part of the work were to review the existing diagnostic techniques for the detection of *E. nucleophila* and *C. molnari*, to highlight fields of application and to point out the major deficiencies of each available method.

Given available diagnostic tools unsuitability for a deeper understanding of fish-parasite relation, the work also aimed at developing more effective diagnostic techniques, which led to the validation of a new in situ hybridization assay for the detection of *E. nucleophila* developmental stages within host tissues.

Moreover, this work aimed at studying the distribution of *E. nucleophila* and *C. molnari* within gilthead sea bream Mediterranean aquaculture and at individuating the major risk factors for the detection of the parasites within different cultural systems.

To serve these purposes, data from a survey covering the whole production cycle of gilthead sea bream were analysed employing different statistical approaches.

As regarding fish-borne zoonotic parasites, the research activities focused on the development of molecular diagnostic assays for the detection and genetic characterisation of zoonotic parasites from freshwater environments.

First, the research activities in this field aimed at the achievement of the first molecular characterization of *Eustrongylides excisus*, an emerging zoonotic nematode from freshwater environments, and at studying this nematode's epidemiology in Central Italy lakes.

The other consistent section of the scientific work within the field of fish-borne parasites aimed at developing an innovative molecular-based diagnosis assay for the

simultaneous identification of the infectious stage of five zoonotic trematodes belonging to the families Opisthorchiidae and Heterophyidae.

The statistical analysis and risk factors individuation studies which brought an indispensable contribution to the study of the epidemiology of the parasites presented in this work was carried out in collaboration with the Norwegian Veterinary Institute (Veterinærinstituttet), Oslo (Norway).

The research activities at the Veterinærinstituttet were supported by Project "Marco Polo" (awarded in 2020), which aims at supporting European researchers' activities at foreign institutes.

Most of the research activities were framed in the European Horizon 2020 projects ParaFishControl "Advanced Tools and Research Strategies for Parasite Control in European farmed fish" (https://www.parafishcontrol.eu/) and PerformFISH "Consumer Driven Production: Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain" (http://performfish.eu/), in which the Department of Veterinary Medical Sciences of the University of Bologna is partner.

2. Cultured Gilthead sea bream relevance in Mediterranean and Italian aquaculture

2.1 International context of aquaculture

Aquaculture is one of the fastest growing food producing sectors in the world and is an increasingly important contributor to global food supply and economic growth. According to the latest worldwide statistics on aquaculture compiled by FAO (FAO, 2020), world aquaculture production reached an all-time record peak of 114.5 million tonnes in live weight in 2018.

The farming of aquatic animals in 2018 was dominated by finfish (54.3 million tonnes), harvested from inland aquaculture (47 million tonnes) as well as from marine and coastal aquaculture (7.3 million tonnes). World aquaculture production of farmed aquatic animals grew on average at 5.3 percent per year in the period 2001–2018, but the growth rated only 4 percent in 2017 and 3.2 percent in 2018 due to a slowdown in Chinese productions, the largest aquaculture world producer. Thus, the combined production from the rest of the world still enjoyed moderate growth of 6.7 percent and 5.5 percent, respectively, in the same two years (FAO, 2020).

2.2 The structure of EU aquaculture sector

(Notes: Data from European Aquaculture Production Report 2014-2019, published by the Federation of European Aquaculture Producers (FEAP) had been collected and analysed before Brexit, thus the report included United Kingdom. Other non-EU countries such as Norway and Turkey are reported.

Scientific, Technical and Economic Committee for Fisheries (STECF) data were published before Brexit, thus the report included United Kingdom. Since Norway is a non-EU country it is not included in the report).

European aquaculture has been sharing the sector's worldwide ascending trend since its production has increased by 24 percent from 1990. As EU capture fisheries production has been showing a decreasing tendency from 1990 to 2016, aquaculture has acquired more relevance in supplying the seafood market throughout the last three decades.

Aquaculture fish production in the 28 EU Member States, Norway and Turkey reached almosto 2.6 billion tonnes in 2019 (FEAP, 2020). As it concerns the dispersal of aquaculture within the 28 EU member countries, the sector shows an extremely unequal geographical distribution (Figure 1), since five countries accounts for three quarters of the overall production. Spain is the largest aquaculture producer in the EU covering 21 percent of the production volume, followed by France (15percent) the United Kingdom and Italy (both with 14 percent) and Greece (with 10 percent). Last reports on aquaculture volumes of production ithin EU countries sostantially confirmed this partioning, with Greece ranking second, just after United Kingdom (FEAP, 2020).

These five countries account for 74 percent of the total EU aquaculture production volume. In terms of value, United Kingdom is the largest contributor in EU with 21

percent of the total, followed by France (16 percent), Spain (13 percent), Greece (12 percent) and Italy (11 percent). These five countries combine 73 percent of the total EU aquaculture value (STECF, 2018) (Figure 2).

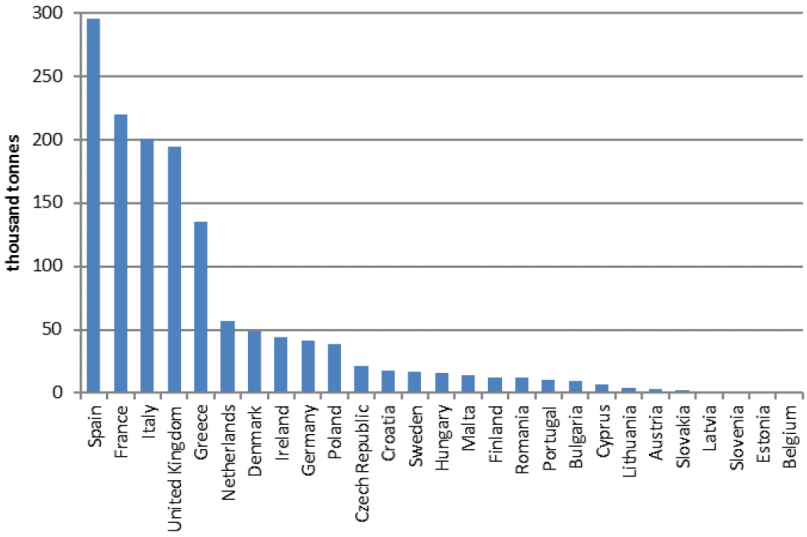

Fig.1. EU aquaculture production in terms of weight (tons) (STECF, 2018).

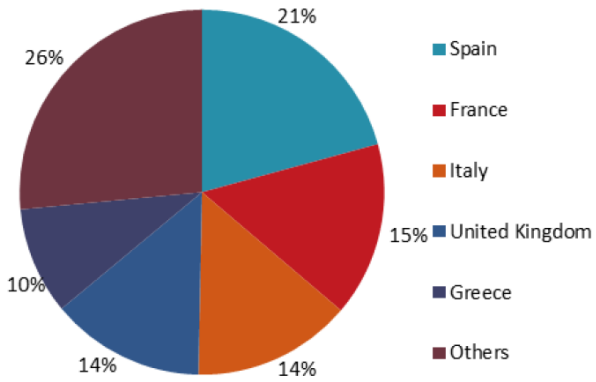

Fig.2. EU aquaculture production in terms of value (STECF, 2018).

In 2016, the aquaculture sector provided 20 percent of the fish and shellfish supply in the EU market. In 2016, marine fishes, freshwater fishes and shellfish accounted for 31, 22 and 47 percent of the EU production of aquaculture in terms of weight, respectively.

In value terms, marine fishes, freshwater fishes and shellfish accounted for 55, 21 and 23 percent of the production value. Despite a less counting production in terms of weight, marine fishes are the most important share in terms of economic value for European aquaculture (STECF, 2018).

The most produced marine species in terms of sales volume is Atlantic salmon representing 45 percent followed by gilthead sea bream (21 percent) and European

seabass (20 percent). In terms of total sales value Atlantic salmon represents 45 percent followed by European seabass (22 percent) and gilthead sea bream (19 percent) (STECF, 2018).

2.3 Gilthead sea bream

Gilthead sea bream is a finfish belonging to the family Sparidae commonly found throughout the Mediterranean; however, it is less frequent in the eastern and south-eastern Mediterranean and very rare in the Black Sea. It is also found in the Atlantic Ocean from the British Isles to Cape Verde and around the Canary Islands (Spain).

Fig. 3. *Sparus aurata*
http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en

It has an oval body, rather deep and compressed, with a head profile regularly curved with small eyes (Figure 3). The overall colour of the body is silvery-grey; a large black blotch at the origin of the lateral line extends on the upper margin of the operculum, where it is edged below by a reddish area; a golden frontal band exists between the eyes, edged by two dark areas (not well defined in young individuals); the fork and tips of the caudal fin are edged with black.

Gilthead sea bream a benthopelagic (demersal behaviour) species, found in coastal environments, inhabiting seagrass beds, rocky and sandy bottoms, as well as in the surf zone and to depths of about 30 m. Adults may be found up to 150 m deep. The species is euryhaline, often entering brackish waters. It is a sedentary fish, solitary, or forming small aggregations. It is mainly carnivorous (mollusc, particularly mussels which it can easily crush, crustaceans, and fish), but accessorily herbivorous.

As regards its reproductive biology, this species is a protandrous hermaphrodite; the majority of individuals are functional males in the first two years (20–30 cm) and then turn into females (33–40 cm). Spawning typically occurs from December to April, when water temperatures are 13–17◦C. Over a long time, marine fish rearing in the Mediterranean region has been exclusively based on collection of wild juveniles from the sea (Pavlidis and Mylonas, 2011). Gilthead sea bream has been traditionally cultured in Italy for centuries in the northern Adriatic lagoons (known as “valli”) in extensive systems, where juveniles of this species are captured in spring and stocked in the valli.

After the 1960s, the availability of wild juveniles decreased drastically because of overfishing and pollution and lot of efforts have been spent for reaching controlled reproduction since then. The availability of “controlled” reproduction techniques for gilthead sea bream, as well as for European seabass, opened the door toward the industrialization of marine aquaculture in the Mediterranean region. The first hatcheries

faced several problems with broodstock and larval culture. Good success at small-scale reproduction was first obtained in Italy, France, and Israel in 1978–1980, but large-scale production of juveniles was possible only a few years later. At present, broodstock maturation and spawning in captivity is technically feasible and does not present any constraints (Pavlidis and Mylonas, 2011).

2.4 EU gilthead sea bream husbandry

Gilthead sea bream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) are the most commonly produced species in the Mediterranean Sea. In EU countries, gilthead sea bream productions recently surpassed those of European seabass, reaching 93,609 tons in 2018 (FEAP unpublished data, 2019).

World production of farmed sea bream and seabass was 376,984 tonnes valued at 2066 million USD in 2016. The capture sector relevance for these species is really limited as it accounts for less than 4 percent of total volumes. About 95 percent of the production of these two species takes place in the Mediterranean area (Llorente *et al*., 2020).

According to FAO aquaculture production data, the combined global production of European seabass (*Dicentrarchus labrax*) and Gilthead sea bream (*Sparus aurata*) increased from 245.3 thousand tonnes in 2008 to 377 thousand tonnes in 2016.

Leading production countries are Turkey and Greece producing 37 percent and 24 percent of the total volume and 35 percent and 25 percent of the total value in 2016, respectively. In 2018 Turkey and Greece accounted for 47percent and 35percent of total volume of gilthead sea bream production and for 48percent and 29percent of European sea bass total volume of production.

The six largest producing countries, Turkey, Greece, Egypt, Spain, Tunisia and Italy, produced more than 92 percent of the total volume in 2016. Despite the most relevant increase in European seabass and gilthead sea bream production since 2008 was scored by Turkey, Egypt and Tunisia, EU most important producers (Greece, Spain and Italy) have moderately increased production volume during the same period by 6percent, 10percent and 12percent, respectively (STECF, 2018).

As concern production figures for EU countries, member states produced 82,600tonnes of European sea bass and 93,600 tonnes of gilthead sea bream in 2018. The main European producer is Greece with 45.5 and 61.0 thousand tonnes of European sea bass and gilthead sea bream respectively, produced in 2018. Greece is followed by Spain and Italy with 22.5 and 7.3 thousand tonnes for European sea bass and 14.9 and 9.7 thousand tonnes for gilthead sea bream respectively (FEAP unpublished data) thousand tonnes and 7.6 thousand tonnes, respectively.

2.5 Italian aquaculture

The main farmed species (in terms of weight) are for shellfish the Mediterranean mussels (*Mytilus* spp.) and Venus clams (*Ruditapes philippinarum* and *R. decussatus*) while for the freshwater macro-aggregate the trout (*Oncorhynchus mykiss* and *Salmo trutta fario*) is the first cultured fish. For the sector of euryhaline species the most farmed species is the sea bream followed by seabass. On the hand of value, the most important species is the trout, and the mussel are one of the last species (STECF, 2018). Italian aquaculture has experienced a decline in production after 2016, sharing the trend of worldwide aquaculture (FAO, 2020). Despite this, sea bream production remained stable and recorded a slight growth trend, going from 7,600 full tons produced in 2016 to 9,700 produced in 2018 (FEAP, 2019) Data are summarized in Table 1.

Table 1: Italian aquaculture production (in tons) and corresponding value (in thousands of euros) in 2018. Source: http://www.api-online.it/index.cfm/it/informazioni/piscicoltura-italiana/

Species	Marine and inland farming sites (tons)	Coastal and lagoon farming sites(tons)	Total (tons)	Value (thousands of euros)	FEAP 2019 total (tons)	FEAP 2019 Value (thousands of euros)
European seabass (*Dicentrarchus labrax*)	6,900	400	7,300	59,000	7,400	40,000
Gilthead sea bream (*Sparus aurata*)	**9,300**	**400**	**9,700**	**75,000**	**9,500**	**90,000**
Shi drum (*Umbrina cirrosa*)	100		100	750		
Eel (*Anguilla anguilla*)	600	250	850	9,400	800	
Mullet (*Mugil cephalus*)		2,500	2,500	9,450		
Trouts (*Oncorhynchus mykiss, Salmo trutta fario*)	37,500		37,500	120,000	33,800	100,724
Salvelinus (*Salvelinus fontinalis*)	800		800	3,600		
European catfish (*Ameiurus melas*)	450		450	2,700	450	2,700
European carp (*Cyprinus carpio*)	600		600	2,600	550	2,365
Sturgeon (*Acipenser sturio*)	1,000		1,000	7,000	900	
Others	1,500		1,500	11,200		
Total	58,750	3,550	**62,300**	**300,700**		

2.6 Italian gilthead sea bream aquaculture

Italian gilthead sea bream aquaculture can be divided into two main farming systems, as for other euryhaline fish species: extensive pisciculture (ponds and confined coastal lagoons) and intensive pisciculture (land-based, inshore and offshore cages). Traditionally, gilthead sea bream was cultured extensively in coastal lagoons and saltwater ponds, until intensive rearing systems were developed during the 1980s.

The Italian "vallicoltura" is a fish rearing system which is based on a natural fish trap, taking advantage of the natural trophic migration of juveniles from the sea into coastal lagoons. Artificial breeding was successfully achieved in Italy in 1981-82 and large-scale production of gilthead sea bream juveniles was definitively achieved in 1988-1989 in Spain, Italy and Greece (FAO, 2005). The hatchery production and farming of this fish is one of the success stories of the aquaculture business. This species very quickly demonstrated a high adaptability to intensive rearing conditions, both in ponds and sea-cages facilities.

2.6.1 Extensive rearing system

This system is based on the natural migration of euryhaline fish, when the fish may be caught, generally in typical fishing traps. Since this practice provides a very limited and unpredictable source of natural juveniles, many modern commercial extensive production units rely on both wild-caught and hatchery-reared juveniles.

Generally, 2-3 g gilthead sea bream are seeded into the lagoons in April-May. Under these systems fish reach the first commercial size (around 350 g) in 20 months and are often farmed together with mullets, eels and European seabass. During the production cycle the fish feed on natural lagoon resources and no supplementary food is provided (FAO, 2005).

2.6.2 Intensive rearing system

Intensive rearing system is characterised by separate production steps, which usually take place in different facilities. Reproduction and larval rearing phases are carried out in hatcheries, which are land-based facilities usually located in coastal areas.

Pre-growing phase take place into indoor or outdoor ponds which are often located within the hatchery farming site area. Gilthead sea bream ongrowing phase may be carried out in land-based concrete ponds or in sea cages close to the coastal line (floating cages) or into off-shore sites (semi-submersible or submersible cages).

Ongrowing sites may be seeded with juveniles purchased from other companies' hatcheries, but large production units normally rear their own. Under excellent environmental and sanitary conditions, pre-fattened juveniles (5 g) reach first commercial size (350-400 g) in about one year. The whole production cycle length ranges between 16 and 18 months normally. Ongrowing in sea cages is simple and economical and it is the fattening system normally used in the Mediterranean basin.

3. Major transmissible diseases in gilthead sea bream farms in the Mediterranean area: most relevant epidemiological, clinical and economic aspects of viral, bacterial and parasitic diseases.

As well as in livestock production, the aquaculture sector is vulnerable to exotic, endemic and emerging diseases (FAO, 2018). The rapid development of mariculture has been associated with the emergence of infectious diseases. Fish farming practices create conditions that can increase the risk of diseases outbreaks by providing high densities of fish, repeated introduction of naive hosts, homogeneous host populations, fast growth and a potential decrease in genetic diversity (Nowak, 2007).

Diseases outbreaks may have multiple and considerable environmental, social and economic impacts in aquaculture. The major consequences of transmissible disease outbreaks in pisciculture include direct costs of lost production due to mortalities and slow growth, as well as temporary or permanent closure of aquaculture facilities, causing loss of employment in aquaculture and related upstream and downstream industries. Furthermore, they can lead to large scale economic losses due to bans on exportation and considerably reduced domestic sales due to public concerns over the safety of consuming fish products (FAO, 2020).

In order to gather the available information about the main sanitary issues in Mediterranean gilthead sea bream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) husbandries, a survey involving gilthead sea bream and sea bass farms across the whole Mediterranean basin has been recently published (Muniesa *et al.*, 2020).

The results of this work report mortality rates, diseases occurrence and distribution based on farmers own recorded data of existing diseases at a regional scale and their impact on production. According to the farmers' data, bacterial infections dominated the reports (75.0 percent) for sea bass, while parasitic infections (57.0 percent) were the most frequently reported infections in gilthead sea bream. The most reported pathogens from hatchery sector were *Vibrio* spp. and viral encephalopathy and retinopathy viral nervous necrosis virus (VER-VNN virus), whereas parasitic infections due to *Sparicotyle chrysophrii* and *Enteromyxum leei,* and the Winter Syndrome, a metabolic disease with multifactorial aetiology, were the most frequently recorded diseases from ongrowing stage. Basing on this survey, the diseases reported to be the major sanitary issues in gilthead sea bream Mediterranean farms are going to be briefly illustrated in the following paragraphs, with particular focus on parasitic diseases.

3.1 Most relevant viral and bacterial diseases in gilthead sea bream Mediterranean aquaculture

3.1.1 Viral Encephalopathy and Retinopathy/Viral Nervous Necrosis (VER/VNN)

Betanodavirus or nervous necrosis virus (NNV, also known as the causative agent of viral encephalopathy and retinopathy) is widely acknowledged as one of the main causes of economic losses in Mediterranean aquaculture. NNV is a RNA-virus belonging to the family of *Nodaviridae* whose genome consists of two segments named RNA1 (3.1

Kb) and RNA2 (1.4 Kb). At present, *Betanodaviruses* are classified into four species, RGNNV, SJNNV, BFNNV, TPNNV and two types of reassortants (namely RGNNV/SJNNV and SJNNV/RGNNV) (Toffan *et al.*, 2017). The disease has been reported in more than 50 fish species of marine fish and in few freshwater farms worldwide (OIE, 2019); as for Mediterranean aquaculture VNN has been recorded and isolated from outbreaks and as well as from asymptomatic European seabass from the 90's and, more recently, from gilthead sea bream (Toffan *et al.*, 2017). In particular, VNN disease outbreaks reported in gilthead sea bream have been related to the reassortant genotype RGNNV/SJNN (Toffan *et al.*, 2017).

These outbreaks occurred at lower temperatures than those observed for seabass (19°C-21°C) and mainly involved larvae, post-larvae and juveniles (Padrós *et al.*, 2020; Volpe *et al.*, 2020). Recently an outbreak involving gilthead sea bream and European seabass in the same farm at the same time has been reported from Italy (Volpe *et al.*, 2020), thus the possibility of the same reassortant genotype transmission between different host species cannot be ruled out. *Betanodavirus* replicates only in the brain, spinal cord and retina, where it causes the necrosis of the nervous cells, and consequently the typical abnormal swimming behaviour. Clinical signs include apathy alternated to swirling and spinning movements, swim bladder hyperinflation, and blindness. Congestion and erosion of the head and nose, darkening and anorexia are also often present (Toffan *et al.*, 2017).

Mortality rates can reach 100% in European seabass larvae, while in older fish the disease is generally less severe. Stressors (i.e. feeding, sorting, netting, etc.) can increase the severity of the clinical signs and cumulative mortality. Survivors remain persistently infected and can transmit the disease to healthy fish for long time (Zrncic *et al.*, 2020). Currently, no vaccine is registered for gilthead sea bream (OIE, 2019).

3.1.2 Lymphocystis disease (LCD)

The lymphocystis disease (LCD) is a highly contagious viral infection responsible for high economic losses in the aquaculture industry worldwide and has been described in more than 125 species of fish from both marine and freshwater environments (Borrego *et al.*, 2017). The causative agent is the lymphocystis disease virus (LCDV), a double-stranded DNA virus which belongs to the genus *Lymphocystivirus*, family *Iridoviridae*. The genotype isolated from gilthead sea bream is LCDV-Sa (López-Bueno *et al.*, 2016) and it has been reported mainly from hatcheries (Muniesa *et al.*, 2020).

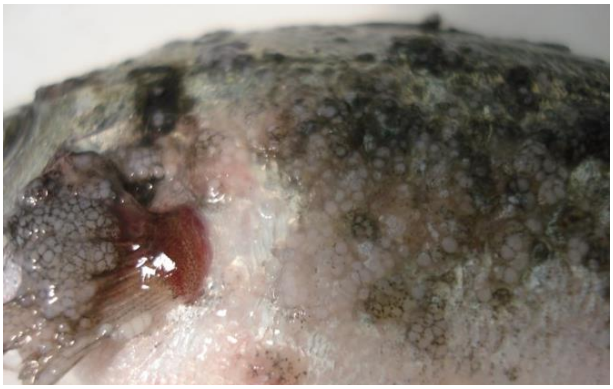

Fig. 4. Lymphocystis disease in gilthead sea bream (Fish Pathology Lab, DIMEVET)

LCD is characterized by the appearance of small nodular lesions on the fish skin and fin (Figure 4),

consisting of an LCDV-infected cell, named lymphocyst or lymphocystis cell. These hypertrophied cells may occur singly or grouped in tumour-like clusters (Borrego *et al.*, 2017). The virus may be hosted for long periods of time by asymptomatic carriers until a decrease in host immunocompetence leads to the virus activation (Carballo *et al.*, 2020).

Diseased fish show low growth rates, which may be caused by the anaemia generally associated with this disease. Mortalities are typically limited to those individuals whose swimming, breathing or feeding is severely impaired by particularly large and cumbersome growths of infected cells. In fish farms, LCD outbreaks may favour secondary bacterial infections, cannibalism and/or parasitic infections, factors that may increase mortality rates (Borrego *et al.*, 2017).

In spite of being a self-limiting benign process, LCDV infection has a high economic impact in hatcheries due to the important delay in fish growth, reducing feed conversion rates and making fish unavailable for commercialization due to external lesions and the lack of acceptance by the consumers (Carballo *et al.*, 2020).

3.1.3 Vibriosis

The word "vibriosis" is commonly used to refer to bacterial diseases affecting wild and farmed fish caused by members of the *Vibrionaceae* family. *Vibrio* spp. are ubiquitous in the marine environment, particularly in tropical and temperate waters especially in the Mediterranean basin.

The genus *Vibrio* includes some species that behave as opportunistic pathogens and can be involved in cultured gilthead sea bream outbreaks (Scarano *et al.*, 2014). Among the most commonly isolated marine *Vibrio* species, *Vibrio anguillarum and Vibrio harveyi* (syn. *V. carchariae*) have been reported from cultured gilthead sea bream from the whole Mediterranean basin (Firmino *et al.*, 2019; Scarano *et al.*, 2014), but several other species have been isolated in farmed gilthead sea bream in the Mediterranean area as well (Arab *et al.*, 2020; Arahal *et al.*, 2016; Tarazona *et al.*, 2015).

In intensive culture systems vibriosis is characterized by systemic haemorrhagic septicaemia with marked abdominal swelling and accumulation of viscous yellowish bloody fluids in the intestine and gall-bladder. Internally, congested blood vessels, branchial, hepatic and intestinal hemorrhages and ascites are the most common histopathological signs in affected fish (Firmino *et al.*, 2019). Vibriosis may interest all size fish, from juveniles to adults. It is worth noting that, despite carrier fish survive the infection, market-size specimens commonly display lesions which make them not suitable for sale, contributing to considerable economic losses (Firmino *et al.*, 2019). Although gilthead sea bream may display clinical signs of vibriosis, including mortality, it often behaves as an asymptomatic carrier, especially with regard to *V. harveyi* infections.

Fish vibriosis can also be a threat for Public Health since species isolated from cultured gilthead sea bream showed antimicrobial resistance to commonly used drugs, such as amoxicillin, ampicillin and other β-lactamics (Scarano *et al.*, 2014).

3.1.4 Photobacteriosis

Photobacteriosis (also known as pasteurellosis or pseudotuberculosis) is a severe bacterial septicemic disease which affects a wide range of piscine hosts worldwide. The causative agent of photobacteriosis is *Photobacterium damselae* subsp. *piscicida* a Gram-negative bacterium which can affect a large variety of marine fish, including gilthead sea bream (Essam *et al.*, 2016).

Photobacteriosis is a temperature-related disease: the onset of the disease typically occurs at temperatures higher than 18ºC (particularly between 25°C and 30ºC) and the majority of the outbreaks have been reported during summer or early autumn (Padrós *et al.*, 2020). Sea bream is susceptible when very young or around the weaning stage of juveniles and remains very sensitive until the size of 6 g. Its susceptibility gradually decreases from then onwards, thus, for this species, photobacteriosis is mostly a problem in the hatchery/nursery and during the first months in the grow-out facilities, especially when the transfer to cages coincides with the warm season (Zrncic *et al.*, 2020).

Acute onsets are hardly accompanied with clinical signs: convulsive erratic swimming prior to death often comprise the only clinical signs of the acute outbreaks, where internal lesions are often absent at necropsy. Anorexia, lethargy, darkening and ulceration of the skin follow shortly afterwards. The chronic form of the disease is characterized by pseudotubercular nodular lesions mainly in the spleen and/or kidney parenchyma (Figure 5) (Zrncic *et al.*, 2020). Several commercial vaccines are available for controlling Photobacteriosis in gilthead sea bream farms (Padrós *et al.*, 2020).

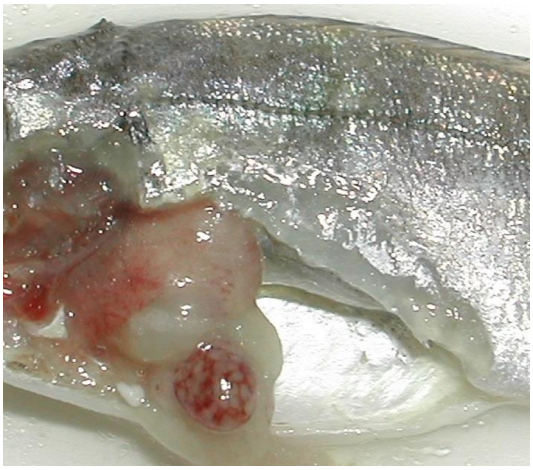

Fig. 5. Gilthead sea bream affected by pasteurellosis (Fish Pathology Lab, DIMEVET).

3.2 Parasitic diseases

Among pathogens commonly reported from cultured fishes, parasites represent significant threats to efficient aquaculture and their control in culture systems is of paramount importance (Timi and MacKenzie, 2015).

Economic losses related to parasites presence in aquaculture accrue not only from mortalities but also from impacts on growth and food conversion, post-harvest downgrading or rejection of carcasses and derived products, fish escapes, management decisions that impact on profitability and the costs and effects of particular husbandry and management practices, such as fallowing, grading, treatment and stock handling (Shinn *et al.*, 2015).

Parasitic diseases control is utterly challenging, since their aetiology is frequently complex and involves, in addition to the parasite itself, environmental and host-related

cofactors and human safety regulations (Fioravanti *et al.*, 2020a). Furthermore, parasites easily develop resistance to frequently applied chemical treatments and vaccines development poses specific issues. Perfect paradigm of what has been said is the impact of salmon lice (*Lepeophtheirus salmonis* and *Caligus* spp.) in Atlantic salmon husbandry, whose control is of key relevance for further growth of the industry (Cerbule and Godfroid, 2020).

Parasitic diseases constitute a key constraint to production, economical sustainability and environmental impact of gilthead sea bream husbandry as well.

3.2.1 Sparicotlylosis

Sparicotyle chrysophrii is a monogenean microcotylid gill parasite affecting several sparid species, in particular gilthead sea bream, and it is currently one of the most important parasitic problems in finfish farming across the Mediterranean (Padrós *et al.*, 2020).

S. chrysophrii is hermaphrodite and has a direct life cycle. Post-larval stages, juveniles and adults live in the gills of the fish and bundles of eggs are released by gravid adults into the environment. Each egg has two polar filaments (the distal filament with a hooked end) which let the eggs attach to natural structures or submerged objects in the aquatic environment. Oncomiradicia hatch after 5-14 days and actively swim in the water column to find a new host (Padros *et al.*, 2020) (Figures 6 and 7).

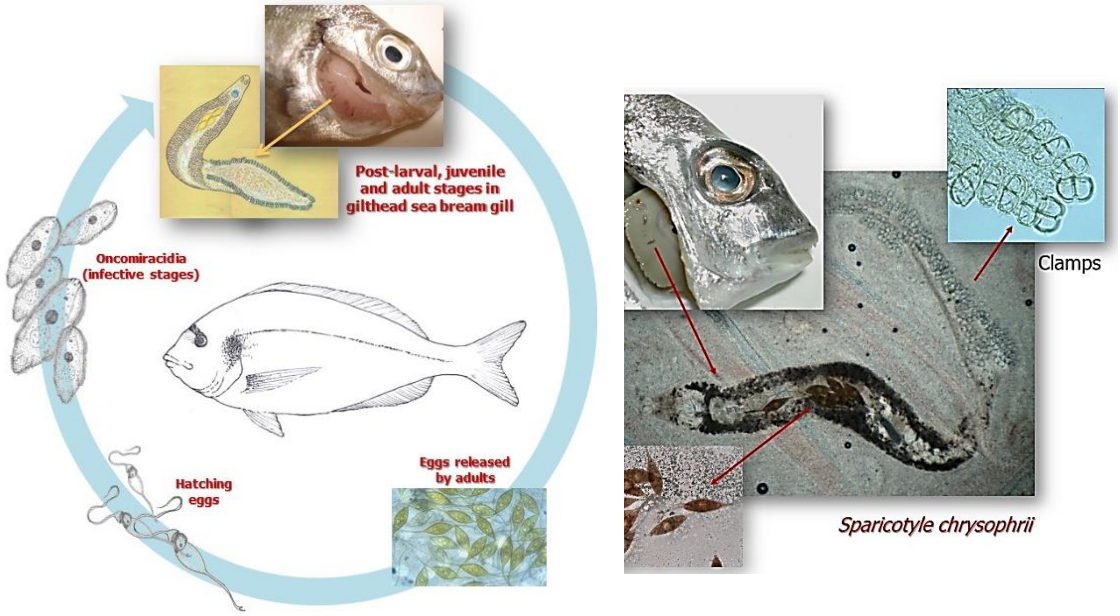

Fig 6 – 7. Life cycle of *Sparicotyle chrysophrii* (Fioravanti *et al.*, 2020a).

Juveniles from nursery or pre-on-growing usually are not infected thanks to the specific rearing system, with complete physical separation of the production batches and

the use of good water quality. Fish become infected when they are seed in ongrowing farming sites, typically sea-cages farms in sheltered areas (Fioravanti *et al.*,2020b).

The presence of eggs and/or oncomiracidia in water entering the fish farm is a relevant risk factor in intensive on-growing systems, due to the fish-to-fish transmission and to the high rearing densities. In fish farms, low currents, low distance between the sea bottom and cages, close vicinity of cages with newly introduced fish to cages with adult fish, infrequent changes and cleaning of nets (a substrate for egg entanglement) and infrequent removal of dead fish are all relevant risk factors influencing the parasite load and parasite outbreaks. Transfer of *S. chrysophrii* from wild infected sparids to cultured fish can also occur (Fioravanti *et al.*, 2020a).

The main pathological effects of *S. chrysophrii* are respiratory dysfunctions due to the association of mechanical lesions and mechanical obstacle to water flow between gill filaments due to the presence of flukes massive bodies, and the progressive anemia induced by the hematophagous attitude of the parasite (Fioravanti *et al.*, 2020b).

Other frequently reported clinical signs are reduced appetite up to anorexia, growth retardation, increase of food conversion rates (FCR) and mortality at high infection intensity. Severe clinical pictures can be also observed at low infection intensity (Fioravanti *et al.*, 2020a). Although the parasite is reported throughout the year, prevalence and intensity are generally higher in the warm season in the Mediterranean, except for Corsica where highest values are observed in winter (Fioravanti *et al.*, 2020a).

As vaccines against *S. chrysophrii* are not currently available, direct preventive strategies are essential to control the disease in the culture system since treatment is often not feasible. When authorized, formalin baths are applied (in synchrony with net changing for the best results). However, bath treatments (with formalin, hydrogen peroxide and other substances) are not authorized in several countries (Fioravanti *et al.*, 2020a).

Recently, attention has been focused on more practical and safe control strategies such as the use of feed additives. In particular, caprylic acid alone (200 mg/kg b.w.) or combined with iron (0.2 % of diet) and immunostimulants like mannan oligosaccharides (MOS) (0.4 % of diet) showed a good efficacy in decreasing intensity of adults and juveniles in the gills, although it did not reduce prevalence (Rigos *et al.*, 2016).

3.2.2 Enteromyxosis

Enteromyxum leei is a myxozoan parasite infecting the intestinal tract of several teleost fish species, especially Sparids. In sparids, this disease is also known as “razor blade syndrome” or “knife-syndrome”, due to the appearance of extremely emaciated affected adults. *E. leei* severely affects gilthead sea bream intensive husbandry in Mediterranean and Atlantic farming sites and it led to the stagnation or abandonment of Mediterranean wide-scale production of red porgy (*Pagrus pagrus*) and sharpsnout sea bream (*Diplodus puntazzo*), the most susceptible host (Fioravanti *et al.*, 2020a).

Although myxosporean usually have an indirect life cycle involving two hosts, a fish and an aquatic invertebrate, direct fish-to-fish transmission has been demonstrated in various marine fish species for *E. leei*. *E. leei* developmental stages and myxospores are excreted into the water with faeces or from dead fish. Developmental stages are infective to other fish and thus responsible for the horizontal transmission. Although an indirect cycle involving an invertebrate host may occur in wild environments, horizontal transmission is the most likely route of parasite spreading in intensive aquaculture (Fioravanti *et al.*, 2020b) (Figure 8).

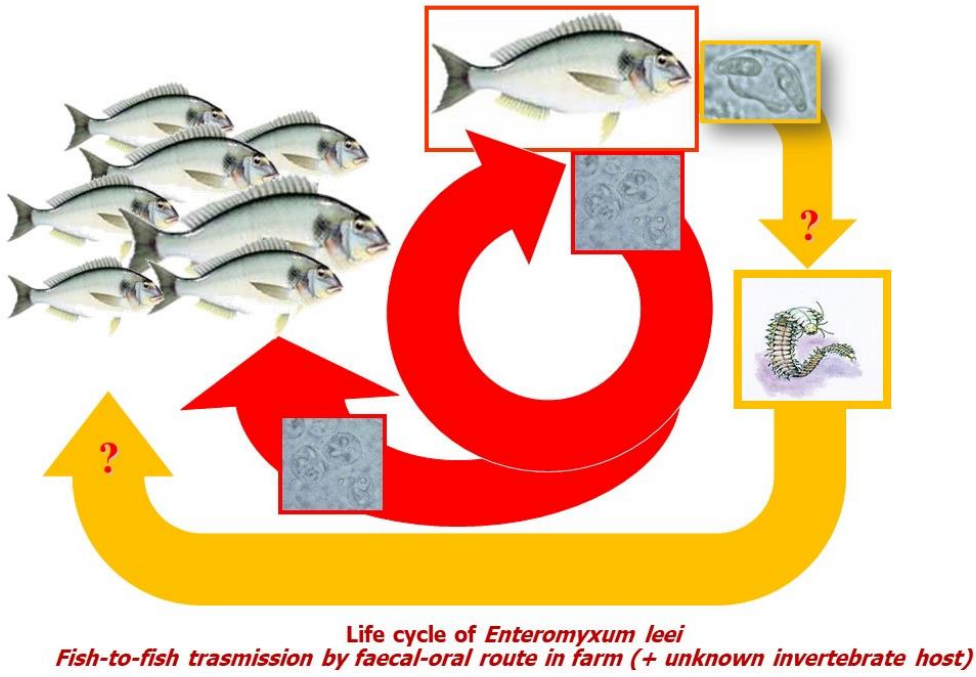

Fig. 8. Life cycle of *Enteromyxum leei*. Courtesy of Prof. Maria Letizia Fioravanti.

In gilthead sea bream farms, factors such as high biomass densities, poor water exchange, recirculation systems and infrequent removal of dead fish may facilitate parasite transmission in cultured stocks (Fioravanti *et al.*, 2020a). Enteromyxosis onset in gilthead sea bream is strictly temperature-related: outbreaks are observed when water temperature ranges from 18° C to 22 °C and outbreaks in some farms have only been observed above 20 °C (Fioravanti *et al.*, 2020a).

Fig. 9. Gilthead sea bream infected with *E. leei* showing severe emaciation (Fish Pathology Lab, DIMEVET).

Both juveniles and adults can be affected, but typical clinical signs are observed in fish >100-150g. Clinical picture is rather aspecific and it is characterized by anorexia and

weight loss until emaciation, which often results in the marked atrophy of epaxial muscle and prominent head bones in severely affected fish (Figure 9). Ascites and abdominal swelling are often observed.

Direct mortality mostly depends on the rearing system and ranges from a low-level dropping mortality in sea cages to heavy sustained losses in raceways or closed systems with heated water. Infection also causes a significant growth retardation and mortality in adult specimens in cage and land-based farms (Fioravanti *et al.*, 2020a). There are currently no registered treatments nor vaccines effective against *E. leei*, thus enteromyxosis control must be carried out controlling risk factors, such as fish biomass or water quality. Some infeed nutraceutical mitigation solutions are currently available, such as sodium butyrate BP-70® (Norel), Sanacore®GM (Adisseo) and Shield™ (Skretting) (Fioravanti *et al.*, 2020a).

3.2.3 *Cardicola aurata*

Cardicola is a genus of digenean trematodes belonging to Aporocotylidae family.

These trematodes infect the blood vascular system of marine and euryhaline teleost fishes, with records from most basins worldwide (Bullard, 2013). They have an indirect life cycle with annelids (not yet identified for *Cardicola aurata*) as intermediate hosts (Figure 10).

In the Mediterranean basin, affected cage-cultured gilthead sea bream display inflammatory reactions and necrosis of the gill tissues due to the presence of parasites eggs in the primary and secondary gill filament (Holzer *et al.*, 2008).

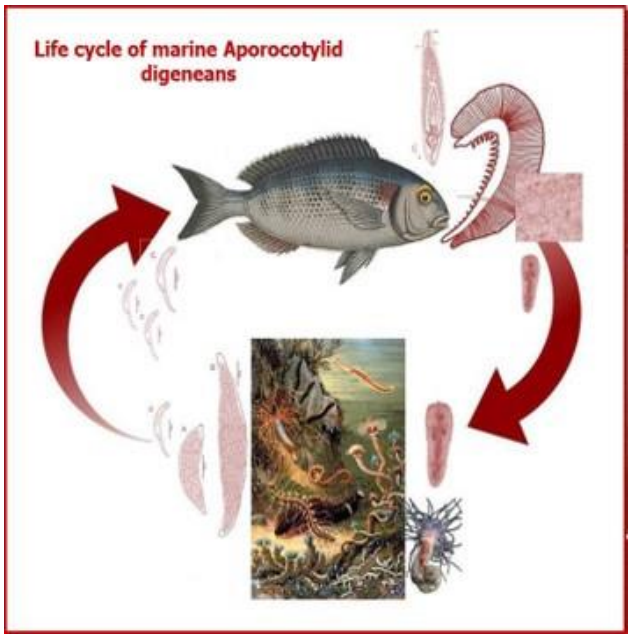

Fig. 10. Life cycle of *Cardicola* spp. Courtesy of Prof. Maria Letizia Fioravanti.

3.2.4 Other parasites

Among other parasites of gilthead sea bream, the ciliate *Cryptocaryon irritans* (Figure 11) and the dinoflagellate *Amyloodinium ocellatum* (Figure 12) can cause disease and mortality in land-based farming systems.

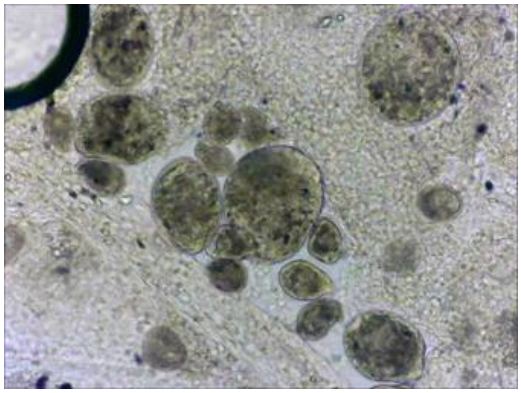

Fig. 11. Trophonts of *Cryptocaryon irritans.* (Fish Pathology Lab, DIMEVET).

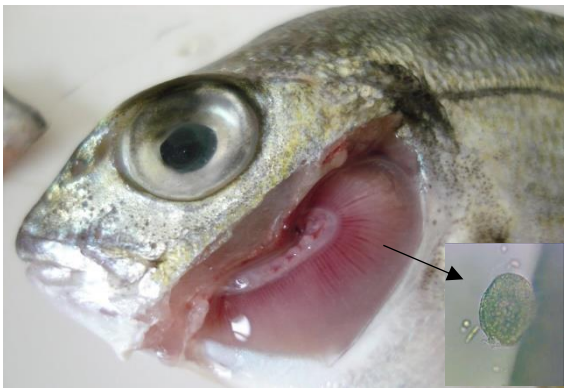

Fig. 12. Gilthead sea bream infected with *Amyloodinium ocellatum* (parasite in the inset) (Fish Pathology Lab, DIMEVET).

Two of the most challenging emerging enteric parasites, the microsporidian *Enterospora nucleophila* and the apicomplexan *Cryptosporidium molnari*, have been reported in gilthead sea bream farmed in the Mediterranean basin, but there is still little scientific knowledge on their biology, epidemiology and impact on sea bream production. As the assessment of presence and distribution of the latter parasites has been the core topic of the research activities treated in this work, the scientific information available is thoroughly reviewed in assigned following chapters.

4. Emerging enteric parasites from cultured gilthead sea bream: biology, epidemiology and clinical aspects of *Enterospora nucleophila* and *Cryptosporidium molnari* infections.

Aquatic animal disease is one of the most serious constraints to the expansion and development of sustainable aquaculture. Globally, a trend in aquaculture is that a previously unreported pathogen that causes a new and unknown disease will emerge, spread rapidly, including across national borders, and cause major production losses approximately every three to five years (FAO, 2020). A long time-lapse (usually years) then ensues, from the time that a serious mortality event is observed in the field, to the subsequent identification and confirmation of its causative agent, to global awareness, and to the establishment and implementation of surveillance and reporting/notification systems and effective risk management measures (FAO, 2020).

This particularly apply to *Enterospora nucleophila* and *Cryptosporidium molnari*, two emerging gastro-intestinal parasites which affect cultured gilthead sea bream *(Sparus aurata*). Contrarily to metazoan parasites, which are very often visible to the naked eye and therefore easily diagnosed, microsporidians and protozoans are few micrometres making their detection hard to achieve.

When unicellular parasites' infections can be observed macroscopically, this is usually due to pathological tissue changes induced by parasites. Despite the limited size of microsporidians and protozoans, their pathogenic effects on fish may be devastating and can negatively impact on fish production (Buchmann, 2015).

4.1 Microsporidia

Microsporidia are obligate intracellular eukaryotic parasites of virtually all animals, including humans, and of some protists such as ciliates and gregarines (Alveolata). There are approximately 1400 described species of microsporidia which are distributed into about 200 genera (Weiss and Becnel, 2014) and they can affect both immunosuppressed and immunocompetent patients, producing sub-clinical to lethal infections.

Microsporidia are characterized by a distinct structure of the spore with its invasion apparatus, an extreme genome compaction and high genetic mutation rates. Microsporidia are highly dependent on their host cell for metabolic processes and energy supply, because of massive nuclear gene losses and reduction of mitochondria to DNA-free mitosomes during their evolution (Corsaro *et al.*, 2019). They take ATP from host cell through bacterial-like nucleotide translocases, which were probably integrated into proto-microsporidians by an ancient horizontal transfer from Chlamydiae (Tsaousis *et al.*, 2008).

Microsporidia infective stage is represented by a unicellular walled spore, which bears a peculiar invasion organ. The invasion apparatus consists of a polar filament

attached to a polar sac, where an anchoring disk and associated membranous structures such as a polaroplast and a posterior vacuole are contained. After an appropriate stimulus, the membranes expand and extrude the filament, which is rapidly everted towards the outside to pierce the host cell plasma membrane or the phagosome, allowing the transfer of the spore content (sporoplasm) (Xu and Weiss, 2005). When entered the host cell membrane, the sporoplasm starts merogonial proliferation, a defined number of asexual binary divisions. The progeny enters sporogony phase, maturation step characterized by the formation of thick-membraned sporonts. Sporonts turn into sporoblasts and sporogony ends with the formation of new spores, whose maturation depends on several factors, such as microsporidia species, host and environment (Vávra and Lukeš, 2013; Han and Weiss, 2017).

Such distinct cytological features together with ultrastructural and developmental characteristics, founded the historical basis of the high-level classification of Microsporidia. The morphological uniqueness of Microsporidia had invalidated any effort of finding clear and affinities and validate relationships with any other lineage, so that their origin and evolution have remained enigmatic for a long time (Corsaro *et al.*, 2019). Thus, debate over placement of the Microsporidia within the tree of life has progressed from historical grouping with spore-forming parasites to the current molecular phylogenetics-based view that they are affiliated with the Fungi.

Various studies have suggested some relationships with different fungal groups, but only recently, it was possible to establish a reliable link with *Rozella*, which are chytrid-like endoparasites of water molds and algae forming the most basal lineage of flagellated fungi (James *et al.*, 2006*;* James *et al.*, 2013). While this recent acknowledgement of a fungal relationship is now accepted by most, their specific relationships and their branching either within the Fungi or outside the group are a topic of further debate (Stentiford *et al.*, 2016; Han and Weiss, 2017).

4.1.1 Fish microsporidia

Of the almost 200 genera described to, half are known to infect aquatic organisms. Excluding genera which infect aquatic life stages of insects, approximately 20 genera infect fish, 50 infect aquatic arthropods, and at least 21 infect aquatic non-arthropod invertebrates, protists, and hyperparasites of aquatic hosts (Stentiford *et al.*, 2013).

Microsporidians occur in hosts inhabiting freshwater, brackish, and marine environments and from a wide array of habitats therein, including temporary water bodies, lakes and rivers, estuaries, rocky shorelines, open ocean, and the deep ocean floor.

A predominant focus on commercially relevant species within the 30.000 extant teleost fish provides the assumption that microsporidian parasites are probably vastly underreported within this group (Stentiford *et al.*, 2013).

Coupling the ability to infect a wide taxonomic range of hosts, and the relative lack of attention applied to pathogen profiling of aquatic organisms, it is apparent that many

thousands of microsporidian taxa remain undescribed in these hosts. Furthermore, the remarkable demonstration of infection capacity across almost all trophic levels within the invertebrate phyla (and indeed within cohabiting aquatic vertebrate hosts) is suggestive that trophic transfer of microsporidians in aquatic environments remains a probable but largely unexplored phenomenon (Stentiford *et al.*, 2016).

Although fish microsporidia can parasitise most organs and tissues, infection due to one species is usually restricted to one organ, tissue, or even cell type (Stentiford *et al.*, 2013). In addition to infections of somatic cells, microsporidians may also specifically infect germinal cells (oocytes, sperm) and be vertically transmitted to offspring (Galbreath Slothouber *et al.*, 2004).

Most microsporidian species known to infect aquatic hosts has the potential to elicit formation xenoparasitic complexes (or xenomas). Xenomas consist in host cells with a completely changed structure due to the presence of the parasites proliferating inside it, both components being morphologically and physiologically integrated to form a separate entity with its own development in the host (Lom and Dyková, 2005). Xenoma formation during aquatic microsporidian parasitism occurs in numerous host taxa and in diverse organ and tissue types. In aquatic hosts, numerous studies report the potential for microsporidian parasites to colonise tissues of commercial importance, such as the musculature of fish and decapod crustaceans (Stentiford *et al.*, 2013). The formerly described microsporidian species from gilthead sea bream have shown intracytoplasmic development and have been associated with the formation of xenomas in skeletal muscle (Abela *et al.*, 1996; Morsy *et al.*, 2013).

4.1.2 *Enterospora nucleophila*

Enterospora nucleophila is a new genus/species of microsporidian belonging to the family Enterocytozoonidae, which includes human and aquatic hosts. Its presence in cultured gilthead sea bream was associated with outbreaks of an emaciative syndrome and was first reported in the early 2000's. However, the challenging diagnosis due to the small dimensions of the spores (1.67µm x 1.05 µm) of *E. nucleophila* probably delayed acknowledgement of its presence and impact and its association with gilthead sea bream emaciative syndrome were not described until recently (Palenzuela *et al.*, 2014).

4.1.2.1 Taxonomy and life cycle

Phylogenetic analysis on *E. nucleophila* SSU rDNA genomic sequence demonstrated its belonging to Enterocytozoonidae family and a strict correlation to *Enterocytozoon bieneusi*, which infects a wide range of vertebrate hosts including humans (Palenzuela *et al.*, 2014). *E. nucleophila* phylogenetic relationships with the closest genera among the phylum are shown in Figure 13.

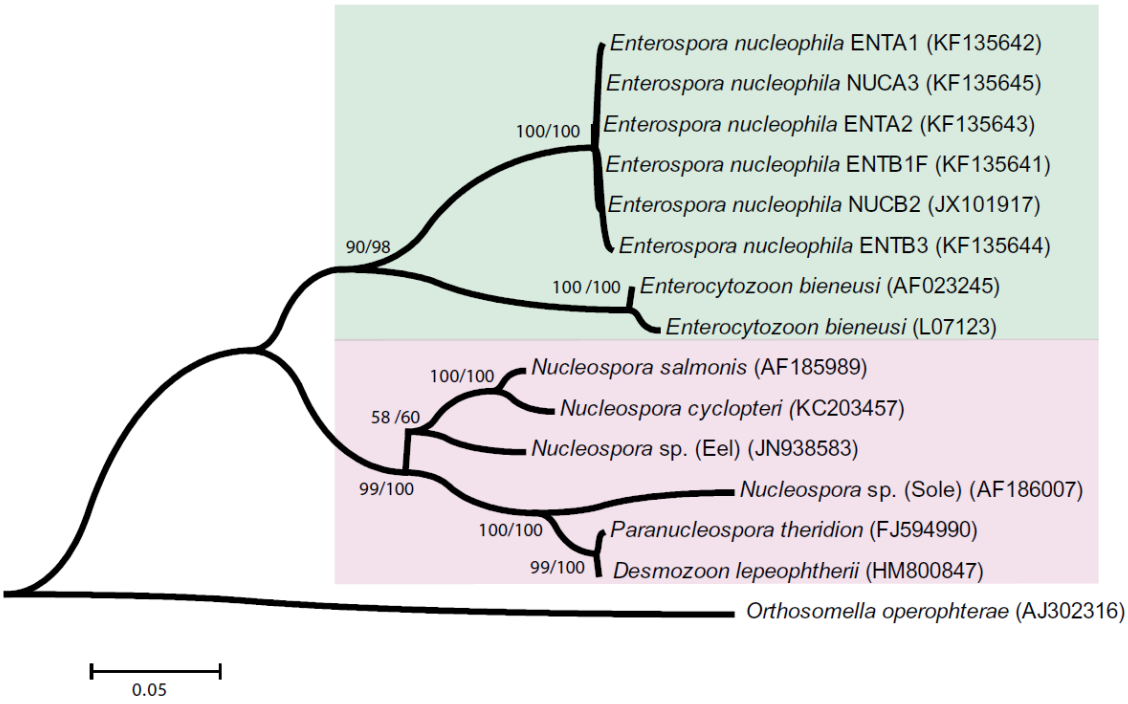

Fig. 13. ssrDNA cladogram of long (>1,000 bp) Enterocytozoonid sequences for which biological and genetic data are available, including *Enterospora nucleophila genotypes*. The topology was inferred using a dataset with 1,280 aligned sites, using maximum likelihood (ML) inference methods, and the tree with the highest log likelihood is shown (ML with a Tamura-Nei model and a discrete gamma distribution with invariant sites). Branch lengths are drawn to scale based on the number of nucleotide substitutions per site. Numbers at the nodes represent branching support by bootstrap values (500 resamplings of this dataset), and Bayesian posterior probabilities. *Orthosomella operophterae* was used as the outgroup. The main clades are shaded in different boxes (from Palenzuela *et al.*, 2014)

E. nucleophila has both intracytoplasmic and intranuclear developmental stages, which is a rarely reported feature within the Phylum. The only three genera sharing this biological characteristic belong to Enterocytozoonidae and all infect aquatic organisms namely *Enterospora*, *Nucleospora* and *Paranucleospora* (formerly *Desmozoon*) (Stentiford *et al.*, 2013).

This microsporidium undergoes intranuclear development in rodlet cells and enterocytes, and cytoplasmic development mainly in enterocytes and macrophages. Up to 16 intranuclear spores result from the sporogonic development of a single plasmodium, whereas more than 40 spores result from several asynchronous reproductive cycles in the cytoplasmic infection (Palenzuela *et al.*, 2014). *E. nucleophila* life cycle and proliferation within both the host cell nucleus and the host cell cytoplasm is shown in Figure 14.

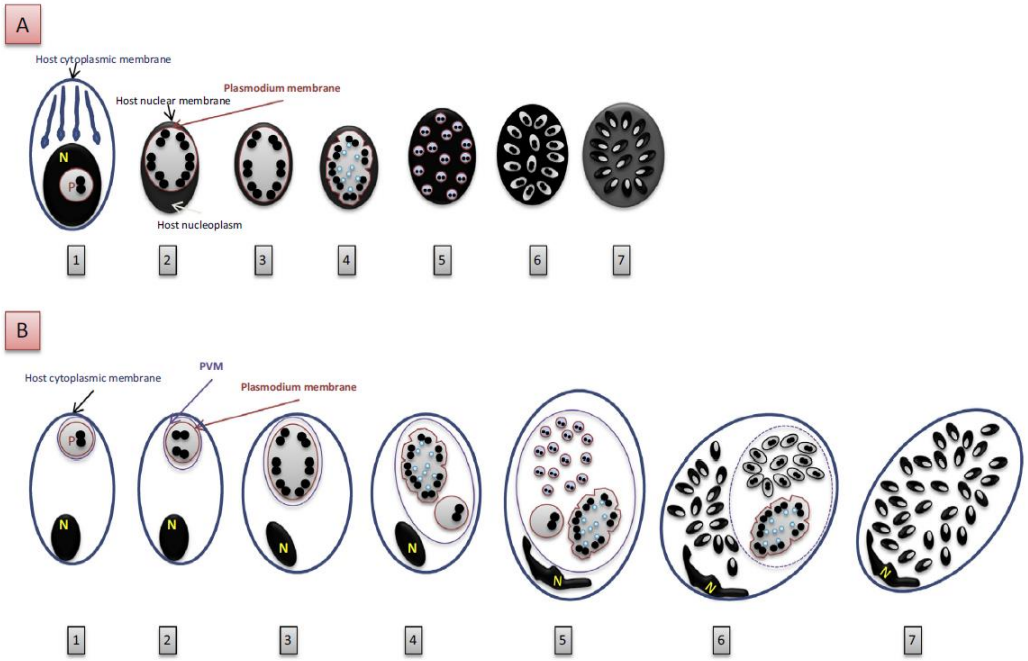

Fig. 14. Diagrammatic interpretation of the intranuclear (A) and cytoplasmic (B) stages of *Enterospora nucleophila*. (A) The infection starts in the host nucleus with an early binucleate meront (1), then merogonial plasmodium (2, 3) with a single plasmodial membrane developed free in the nucleoplasm; diplokarya are visible. The differentiation of the extrusion apparatus occurs before plasmotomy in the sporogonial plasmodium (4); sporoblasts appear and mature (5, 6) and finally spores are visible in the host nucleoplasm (7). (B) The infection starts with an early binucleate meront in the cytoplasm of the host cell surrounded by the membrane of the parasitophorous vacuole (1), which subsequently divides, producing a merogonial plasmodium (2, 3). The division and maturation process are asynchronous with different merogonial and sporogonial plasmodia at different stages (4–6). Finally, multiple spores are found free in the host cytoplasm (7) without any interfacial envelopment. N, host nucleus; P, plasmodium; PVM, parasitophorous vacuole membrane (from Palenzuela *et al.*, 2014).

4.1.2.2 Histopathological lesions associated with *E. nucleophila* in affected gilthead sea bream

In the earliest stages of infection, the parasite replicates in intestine and stomach, whereas it can be mainly detected from hematopoietic organs (spleen and kidneys) and stomach successively. In heavy infections, the intestine displays histological lesions of enterocyte hypercellularity and proliferation of rodlet cells. Infected enterocytes show *E. nucleophila* spores in the cytoplasm, and a pyknotic nucleus, karyorrhexis or karyolysis. Lymphocyte infiltration can be observed at the base of the mucosa and frequent eosinophilic granule cells between enterocytes.

In the intestinal submucosa, macrophage aggregates containing spores are surrounded by lymphocytes and granulocytes, with submucosal infiltration of granulocytes. Furthermore, macrophage aggregates developing into granuloma surrounding necrotic areas with parasite debris can also be observed. Among granulocyte, mast cells are the main type involved in the inflammatory reaction (Picard-

Sánchez *et al.*, 2020). Spores measurements in nuclear and cytoplasmic locations do not vary and they are 1.83 ± 0.15 x 1.11 ± 0.15 µm (Palenzuela *et al.*, 2014).

4.1.2.3 Clinical signs associated with *E. nucleophila* in affected gilthead sea bream

E. nucleophila infection in gilthead sea bream is associated with an emaciative syndrome, whose onset is usually recorded during fish first winter in sea cages. The most common observed clinical manifestation of the infection in affected batches was stunted growth (Palenzuela *et al.*, 2014), with growth arrest and emaciation. The specific growth rate of a naturally infected stock barely reaching 20% of that of a healthy stock of fish of equivalent size and reared under the same conditions (Picard-Sánchez *et al.*, 2020).

The other most reported clinical sign is trickling mortality, with daily mortality rates ranging between 0.1%-0.3% and reaching 1% at peaks. Despite these low figures recorded at farming sites, a recent work reported mortality rates reaching 50% in naturally infected fish which were reared into an experimental facility (Picard-Sánchez *et al.*, 2020). Infected fish exhibited cachexia, emaciation and lethargy and occasional scale loss; cachexia is clearly visible from dorsal view.

Internal organs appear pale and the intestinal walls are often thinned and transparent, and frequently contained clear or greenish fluid and white faeces in the terminal portion. Occasionally, ascites is present (Picard-Sánchez *et al.*, 2020). Sometimes intestinal coinfections with *Enteromyxum leei* or *Cryptosporidium molnari* in gilthead sea bream have been observed (Palenzuela *et al.*, 2014).

4.1.2.4 Host species and geographical distribution

To date, *E. nucleophila* has only been reported from cultured gilthead sea bream. Parasite's records mainly came from the Mediterranean area, more specifically from Spain (Palenzuela *et al.* 2014; Picard-Sánchez *et al.* 2020), Italy (Caffara *et al.*, 2014a; Caffara *et al.*, 2014; Scaturro, 2015; Gustinelli *et al.*, 2016; Mazzone *et al.*, 2019a) and Greece (unpublished data, Palenzuela and Sitjà-Bobadilla). The parasite was also detected from a facility located off of the Atlantic Spanish coast (Palenzuela *et al.*, 2014).

4.1.2.5 Epidemiology

E. nucleophila infections have been reported from on-growing facilities under different rearing conditions. Most of the infected batches were farmed in sea-cages (Palenzuela *et al.*, 2014; Scaturro, 2015; Ahmed *et al.*, 2019; Mazzone *et al.*, 2019a), but *E. nucleophila* has also been detected at land-based earth and concrete ponds facilities (Palenzuela *et al.*, 2014). Mean weight of affected batches ranged between 15 g and 340 g. A three-years survey has been recently carried out in eight farms along two production cycles, covering all the stages from the eggs to the harvest size (unpublished data, Palenzuela and Sitjà-Bobadilla). Unpublished data from this survey show that the first

positive cases were detected at nursery stage, with the smallest fish found to harbour the parasite weighed 0.9 g. At on-growing stage different prevalence rates where observe, with all the sampled cages affected eight months after seeding. A decreasing trend of positivity related to age was also observed, with some cages still testing positive at harvest size. No other epidemiological data on *E. nucleophila* distribution among Mediterranean farms and within gilthead sea bream production cycle are currently available.

4.2 Cryptosporidia

Parasites belonging to the genus *Cryptosporidium* are obligate intracellular protozoan parasites within the phylum Apicomplexa, which includes other significant human pathogens such as *Plasmodium*, *Toxoplasma*, *Isospora*, *Sarcocystis*, *Cyclospora* and *Babesia*. Like the other members of this phylum, *Cryptosporidium* has a complex life cycle with both asexual and sexual stages and invasive stages which display the characteristic apical complex from which the phylum name is derived (Leitch and He, 2011). *Cryptosporidium* spp. infect the microvillus border of the gastrointestinal epithelium of a wide range of hosts, both vertebrate and invertebrate, both terrestrial and aquatic, including humans.

Numerous investigations have been conducted worldwide to shed light on taxonomy, distribution, and epidemiology of *Cryptosporidium* spp.. Because of the morphological similarities among *Cryptosporidium* species, the wide host range of some species and the polymorphism within species, morphological, biological and molecular data are required to assign a *Cryptosporidium* isolate to a given species.

To date, isolates have been assigned to forty-three species using such data, and many more have been assigned to a genotype or subtype based on molecular data (GenBank). Among these species, *C. hominis* and *C. parvum* are the major causative agents of human cryptosporidiosis, the former being predominant in humans and the latter zoonotic and highly prevalent in livestock. Transmission occurs via the oocyst stage shed in faeces, transmitted directly to animals or people, or through the consumption of contaminated water and food (Chalmers *et al.*, 2020). The protozoan is ubiquitous in surface waters and cryptosporidiosis is considered a serious zoonotic waterborne disease (Xiao *et al.*, 2000; Xiao and Feng 2008; Efstratiou *et al.*, 2017).

Cryptosporidiosis begins with the ingestion of fully sporulated and environmentally resistant oocysts. After excystation in the upper small intestine, the released sporozoites penetrate the mucus layer and attach to nearby enterocytes, causing them to form a parasitophorous vacuole around the parasite, which then differentiates into a trophozoite. An unusual feature of this vacuole is that it is located within the host cell plasma membrane, but outside the host cell cytoplasm, separated from the latter by a so-called feeder organelle and a specialized concentration of host cell cytoskeletal elements. Mitotic division of the parasite at this point results in a type I meront and the production of six or eight merozoites. The merozoites resemble sporozoites. They escape

the parasitophorous vacuole and attach to nearby enterocytes, establishing amplified asexual infectious cycles. Alternatively, the merozoite infection may result in a type II meront, and the production of four merozoites. As with the merozoites originating from type meront, type II merozoites escape to infect nearby enterocytes, producing either a macrogamont (female) or a microgamont (male). Sixteen or more microgametes from the microgamont are released and each can fertilize a macrogamont to form a diploid zygote, which differentiates to an oocyst. Meiosis then results in 4 sporozoites being formed. This constitutes the sexual cycle, the end product of which is either a fully sporulated thin-walled oocyst (20%) that excysts within the host and results in autoinfection, or a thick-walled oocyst (80%) that is excreted into the environment (Leitch and He, 2011).

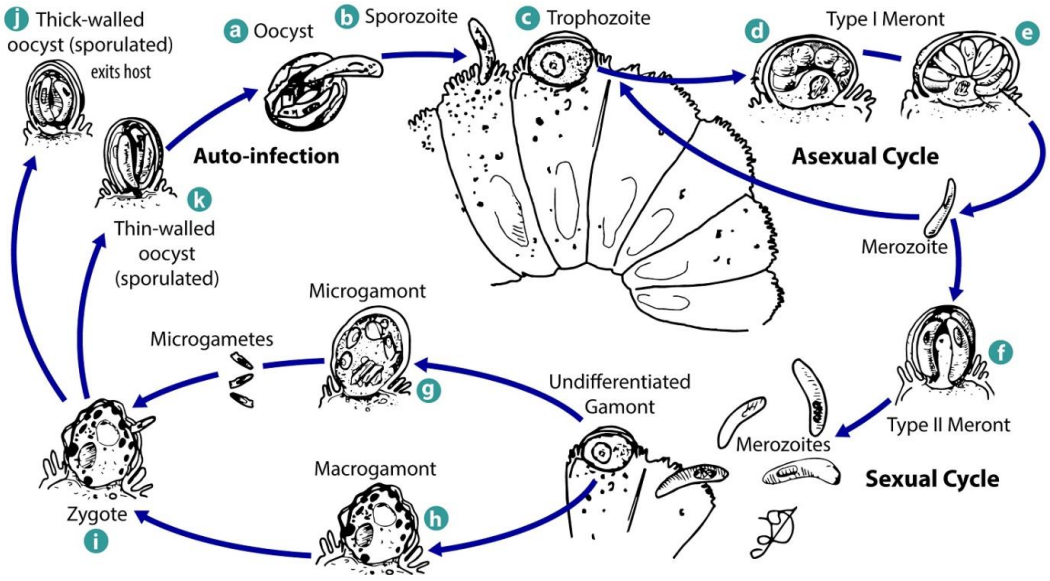

Fig. 15. *Cryptosporidium* spp. life cycle within terrestrial mammal enteric mucosa. Major discrepancies compared to *Cryptosporidium* spp. life cycle in piscine hosts are related sporulation, which occurs deeply into the epithelial layer in piscine hosts, whereas it is epicellular in mammalian hosts. https://www.cdc.gov/dpdx/cryptosporidiosis/modules/Cryptosporidium_LifeCycle_lg.jpg

4.2.1 Fish cryptosporidia

Cryptosporidium has been described both in freshwater and marine organisms and three species of this genus recognise fish as their type hosts: *Cryptosporidium molnari* in gilthead sea bream and European sea bass (*Dicentrarchus labrax*) (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Palenzuela *et al.*, 2010), *Cryptosporidium huwi* in the ornamental fish guppy (*Poecilia reticulata*) (Ryan *et al.*, 2015), and *Cryptosporidium scophthalmi*, which has been reported in turbot (*Psetta maxima*) (Alvarez-Pellitero *et al.*, 2004).

Moreover, several piscine genotypes (piscine genotypes 2–8) and five un-named novel genotypes have been described (Couso-Pérez *et al.*, 2019). Moreover, *Cryptosporidium* usually infecting terrestrial animals have been isolated also from fish, in particular the zoonotic *C. parvum* and *C. hominis* (Ryan *et al.*, 2014). As concerns

biological characteristics, *Cryptosporidium* species reported from piscine hosts share most of the morphological and ultrastructural features of other species from terrestrial hosts (Alvarez-Pellitero *et al.*, 2004; Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Ryan *et al.*, 2015). However, major discrepancies may be found in sporulation, which occurs deeply within the epithelial cell in piscine host, instead of occurring in apical position. Moreover, the accumulation of oocysts in the gastric or intestinal mucosa of aquatic hosts often results in necrosis, vacuolation, and sloughing of epithelial cells. These characteristics contrast with the persistent epicellular disposition of species from terrestrial vertebrates and their mild histological impact within immunocompetent terrestrial hosts (Palenzuela *et al.*, 2010)

4.2.2 *Cryptosporidium molnari*

C. molnari presence in Mediterranean aquaculture was firstly recorded in the early 2000s (Sitjà-Bobadilla and Alvarez-Pellitero, 2001) and, despite the controversies about the existence of a piscine genus separated from that of terrestrial vertebrates (Paperna and Vilenkin, 1996) it was described as a new species within the genus *Cryptosporidium* (Alvarez-Pellitero and Sitjà-Bobadilla, 2002). During the following years, given the growing impact on Spanish aquaculture, other studies were carried out to deepen *C. molnari* transmission routes (Sitjà-Bobadilla and Alvarez-Pellitero, 2003) and epidemiology in different rearing conditions (Sitjà-Bobadilla *et al.*, 2005).

4.2.2.1 Taxonomy and life cycle

C. molnari was erected as a new species in 2001 because of morphological characteristics and, almost a decade later, its molecular identification based on the sequences of the small-subunit ribosomal DNA (SSU rDNA) fragments confirmed this classification. *C. molnari*-like genotypes have also been described from brown trout (Couso-Pérez *et al.*, 2019), and from guppy (Ryan *et al.*, 2004) but their genetic relationship and belonging to *C. molnari* species has yet to be confirmed. *C. molnari* phylogenetic relationships with other Cryptosporidium species isolated both from animal and human hosts are shown in Figure 16.

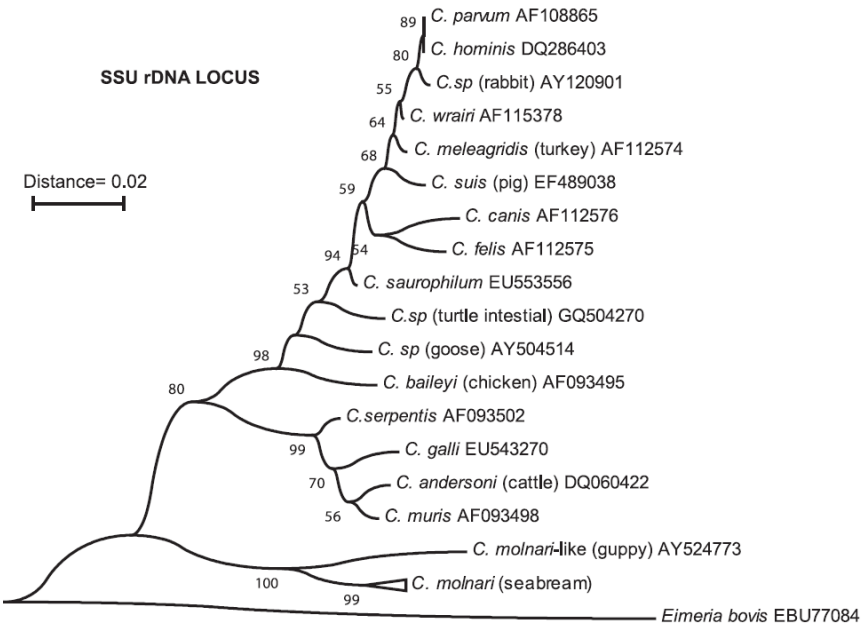

Fig. 16. Cladogram of *Cryptosporidium molnari* and selected *Cryptosporidium* genotypes using neighbor-joining analysis of SSU rDNA sequences, rooted with the coccidian *Eimeria bovis*. The GenBank sequence accession number is provided for each sequence. Genetic distances were computed with the Kimura 2-parameter method with complete deletion of gaps. Percentage bootstrap support values above 50 are shown at the tree nodes. Scale bar represents genetic distance (nucleotide substitutions per site). (From Palenzuela *et al.* 2010).

C. molnari can be transmitted directly through the ingestion of fully sporulated oocysts, like other *Cryptosporidium* species. Transmission may also occur by direct ingestion of infected viscera, since cannibalism is a frequently observed phenomenon among gilthead sea bream within intensive culture conditions (Sitjà-Bobadilla and Alvarez-Pellitero, 2003). After been ingested, the oocysts undergo excystation and release four infective sporozoites. Sporozoites actively penetrate the surface of target cells, mainly from epithelial layer of stomachal mucosa and, less frequently, enterocytes. Both the sexual and asexual phase of *C. molnari* proliferation take place deeply within the stomachal mucosa, leading to the release of fully sporulated oocysts and to a massive damage of the host tissues.

4.2.2.2 Host species and geographical distribution

To date, *C. molnari* has been reported from gilthead sea bream (the type host) and from European sea bass, with more severe pathological consequences within gilthead sea bream farms. Records came from Atlantic and Mediterranean coasts of Spain (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla *et al.*, 2005; Palenzuela *et al.* 2010), and from Italian and Croatian facilities (Mazzone *et al.*, 2019a). Two *C. molnari*-like genotypes has also been reported from a cultured guppy (Ryan *et al.*, 2004) and from a brown trout from Lake Geneva (Swiss) (Couso-Pérez *et al.*, 2019).

4.2.3 Clinical signs associated with *C. molnari* in affected gilthead sea bream

Only gilthead sea bream with high intensity of infection exhibited clinical signs, consisting of whitish faeces, abdominal swelling and ascites (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla and Alvarez-Pellitero, 2003; Sitjà-Bobadilla *et al.*, 2005).

4.2.4 Histopathological lesions associated with *C. molnari* in affected gilthead sea bream

Meronts and gamonts in extracytoplasmic position produce no lesions to the host gastric and enteric mucosa, whereas zygotes and oocysts cause massive necrosis of epithelial cells. In the earlies stages of infection affected cells display marginalized cytoplasm laterally located nucleus. Clusters of oocysts usually occupy wide areas of necrotic epithelium, where debris of initially infected cells can be found; clusters of oocysts within the epithelium may also be surrounded by a vacuolar space. Sloughing of epithelial cells released oocysts into the stomach lumen in heavily infected fish, sometimes in groups. Despite the destructive effect, inflammatory reaction in affected epithelia is generally very poor, with only few phagocytes surrounding oocysts or abundant rodlet cells close to infected areas. Fully sporulated oocysts, measuring 4.72 ± 0.53 x 4.47 ± 0.51 µm can be detected within stomach or rarely within intestine epithelia and in the gastric or intestinal lumen, as well as in faeces (Alvarez-Pellitero and Sitjà-Bobadilla, 2002).

4.2.5 Epidemiology

Transmission of *C. molnari* seems to take place mostly through the water supply, as in other *Cryptosporidium* species. Direct horizontal transmission has also been demonstrated (Sitjà-Bobadilla and Alvarez-Pellitero, 2003). The only existent epidemiological survey regarding *C. molnari* presence within the production cycle of gilthead sea bream stated the presence of the parasite at any rearing stage after post-larval. The highest estimated positivity rates have been observed at pre-ongrowing and ongrowing stages. Most of the ongrowing batches were farmed in sea-cages, but *C. molnari* has also been detected at land-based earth ponds and tanks facilities (Sitjà-Bobadilla *et al.*, 2005). Since fish were always positive after weaning, the authors (Sitjà-Bobadilla *et al.*, 2005) hypothesize that *C. molnari* infects fish at previous production stages (post-larval or nursery), either through water or live food consumption. Other authors experimentally demonstrated that *C. molnari* can infect nauplii of *Artemia* spp. (Méndez-Hermida *et al.*, 2007).

4.3 Research activities on *C. molnari* and *E. nucleophila* occurrence in farmed gilthead sea bream across the Mediterranean area

The research activities on *E. nucleophila* and *C. molnari* mainly aimed at mapping the knowledge on parasites occurrence in gilthead sea bream aquaculture. The first section of the scientific contribution focused on diagnostic tools for the detection of *E. nucleophila* and *C. molnari* in gilthead sea bream. A review of the existing diagnostic techniques for the detection of *E. nucleophila* and *C. molnari*, highlighting fields of application and pointing out the major deficiencies of each available method is provided. Given available diagnostic tools unsuitability for a deeper understanding of fish-parasite relation, the work also provides of a new in situ hybridization assay for the detection of *E. nucleophila* developmental stages within host tissues.

Moreover, this work aimed at studying the distribution of *E. nucleophila* and *C. molnari* within gilthead sea bream Mediterranean aquaculture and at individuating the major risk factors for the detection of the parasites within different cultural systems. To serve these purposes, data from a survey covering the whole production cycle of gilthead sea bream were analysed employing different statistical approaches.

5. Major fish-borne zoonotic nematodes and trematodes in EU Countries.

Foodborne parasitic diseases are a neglected group of zoonoses, whose impact on human health has been underestimated for a variety of reasons (Robertson, 2018). Frequently foodborne zoonotic diseases do not have acute manifestations, but they rather have chronic and insidious impact on the human host, which often leads them to be overlooked. Moreover, foodborne parasitic diseases neglection is due to their perception as an issue related to poverty and confined to underdeveloped countries (Robertson *et al.*, 2020). Population living in areas with a lack in basic infrastructure elements, such as water supply, sanitation, housing, and transport, are more exposed to some foodborne parasites and have higher risks to be infected than population living in developed countries. However, in an increasingly globalised world, with considerable movement of people and animals both between and within countries, as well as an internationalisation of commerce and globalised food supply (Robertson *et al.*, 2014), foodborne pathogens have assessed an increased relevance in European countries (Robertson *et al.*, 2020).

Globalization provides multiple phenomena which may bring about the emergence and re-emergence of foodborne parasites, such as migratory flows, movements of people, creation of international markets letting the movement of live animals and food products become easier and faster than before. A greater variety and quantity of items are shipped to more places today, and greater numbers of people travel greater distances, encountering more people and goods than at any time in history, and thus providing new opportunities for the spread of foodborne parasites. Introduction of livestock, companion animals, aquatic animals, and wild-life to new areas can bring parasites once considered exotic to new locations or can become infected with endemic parasites, despite international regulations or guidelines to minimise such risks (Robertson *et al.*, 2014).

Fish-borne parasitic zoonoses are a wide group of diseases caused by a variety of parasites belonging to several taxa, among which trematodes and nematodes are the most represented (Pham Cu and Murrell, 2020; Rahmati *et al.*, 2020; Simsek *et al.*, 2020). Although caused by extremely different parasites, these diseases share the transmission route to human hosts, which occurs through the consumption of raw or undercooked fish products bearing the infectious stages of the parasites. Affected human hosts may display a wide variety of clinical pictures, ranging from mild to severe gastro-enteric syndromes or hypersensitivity reactions, as for *Anisakis simplex* Rudolphi, 1809 infections (Rahmati *et al.*, 2020; Simsek *et al.*, 2020) or even neoplastic lesions, as for some liver flukes' infections (Myint *et al.*, 2020).

By opening new markets and transforming food consumption patterns, globalisation has also led to rapid and massive changes in people's eating habits. In European countries infections with most of food-borne parasites from imported fish or fish products is a recent, but increasing, phenomenon associated with increased consumption of fish *per se*, expansion of the aquaculture industry, new trends in fish consumption, and development of the cold chain to transport fish around the globe with

insufficient inspection (Robertson *et al.*, 2014). The increasing popularity of raw fish preparations belonging to culinary cultures from all around the world, has led to a dramatic rise in the incidence of numerous fish-borne zoonotic parasitic infections in previously uninfected ethnic groups (Macpherson, 2005).

Because of the spread of risky eating habits, previously restricted to determined geographical areas, to large urban and sub-urban populations, the lack of control in the food chain, a paucity of awareness of the problem among relevant agencies (Figure 17) (Trevisan *et al.*, 2019), an increasing number of people in EU countries is at risk to get infected with fish-borne parasites.

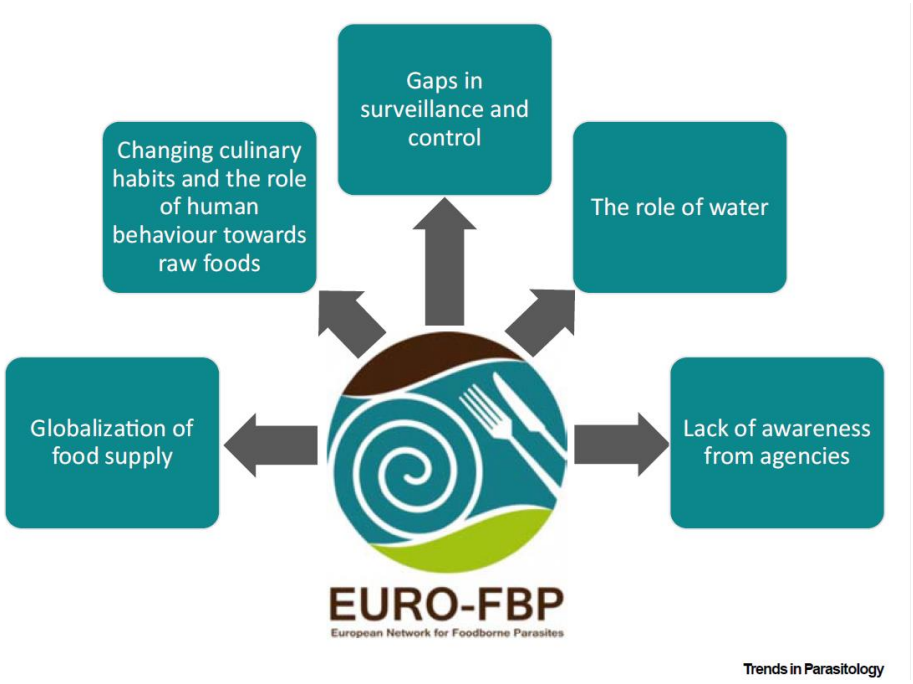

Fig. 17. Top five drivers of foodborne parasitic diseases in European countries (from Trevisan *et al.*, 2019).

Basing on foodborne parasitic zoonoses rankings assessed by EFSA

Basing on the rankings of emerging parasitic zoonoses assessed by the world's most renowned Public Health authorities, fish-borne parasitic diseases emerging as a serious issue for human health worldwide are caused by helminths belonging to the family Anisakidae, Heterophyidae and Opistorchiidae (EFSA, 2010; FAO and WHO, 2014). For this reason, this chapter provides an overview of these zoonotic parasites in EU Countries.

5.1 Fish-borne zoonotic nematodes

Fish-borne nematodes infections in humans are common in countries where people have a traditional custom of consuming live, raw, cold smoked, lightly cooked or marinated fish (Eiras *et al.*, 2016). Several species of fish-borne nematodes are

recognized as causative agents for human diseases: in the family Anisakidae, *Anisakis*, *Pseudoterranova* and *Contracaecum* are well known as human pathogens with cosmopolitan distribution. In the family Gnathostomatidae, several *Gnathostoma* spp. are known to be infective to humans in Asia, South and Central America, and in some areas of Africa (Eiras *et al.*, 2016). In the family Capillariidae, *Capillaria philippinensis* is a fish-borne pathogen related to severe, sometimes fatal, diarrhoea reported from South East Asia (Cross and Belizario, 2007). In the family Dioctophymatidae, *Eustrongylides* spp. and *Dioctophyme renale* are fish-borne zoonotic nematodes seldomly reported from human hosts in different geopgraphical areas (Eiras *et al.*, 2016). Anisakidae infections in humans are the most reported fish-borne parasitic zoonoses from European countries (Mattiucci *et al.*, 2017).

5.1.1 Biology and life cycle of zoonotic nematodes within the family Anisakidae

Anisakidae life cycle is graphically summarized in Figure 18. Nematodes belonging to the genus *Anisakis* are characterized by an indirect life cycle, with a definitive, an intermediate, and several paratenic hosts. Definitive hosts for *Anisakis* spp. are represented by several species of cetaceans (Aibinu *et al.*, 2019), whereas various species of piscivorous birds and pinnipeds associated with freshwater, brackish and marine environments (such as cormorants, pelicans and seals) can play this role for *Contracaecum* spp. (Shamsi, 2019). As concerns *Pseudoterranova* spp., definitive host role is played by a variety of pinnipeds, such as seals, walruses and sea lions (Hochberg *et al.*, 2000).

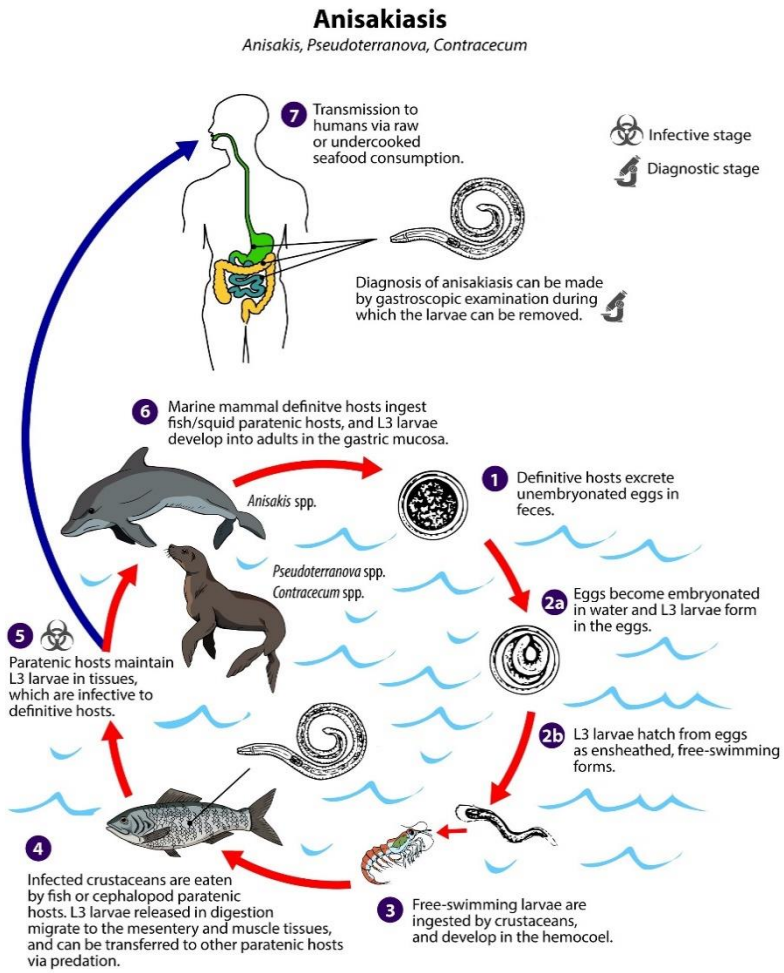

Fig. 18. Anisakidae life cycle.https://www.cdc.gov/dpdx/anisakiasis/modules/Anisakis_LifeCycle_lg.jpg

Adult nematodes parasitize definitive hosts' gastro-intestinal tract, where they reproduce, and eggs are shed into marine environment with definitive hosts' faeces. When they reach water, unembrionated eggs develop the first stage larvae (L1), which undergo two molts inside the eggs and hatch as third stage larvae (Baird *et al.*, 2014; Buchmann and Mehrdana, 2016). Third stage free-swimming larvae (L3) are ingested by the intermediate hosts, represented by crustaceans (copepods, decapods, isopods, amphipods, euphausiids) and molluscs for some species (Baird *et al.*, 2014). Because of its ubiquity within marine environments worldwide (Siegel, 2000) the most represented first intermediate hosts in *Anisakis* life cycle are small crustaceans belonging to the order Euphausiacea ("Krill") (Smith and Wootten, 1978). In these intermediate hosts L3 develop into infective L3, which are further transferred to several paratenic hosts, such as planktophagous fish, piscivorous fish and cephalopods (Aibinu *et al.*, 2019).

In the paratenic hosts L3 actively migrate from the intestine to abdominal cavity and encapsulate in the visceral without developing any further. The life cycle comes to a completion when infected crustaceans, fish or squid are eaten by marine mammals such as whales, seals and dolphins or piscivorous birds. In these definitive hosts L3 larvae

molt to fourth stage larvae (L4) and subsequently to the adult stage in the gastro-intestinal tract (Ivanović *et al.*, 2017). As concerns anisakiasis as a zoonotic disease, humans get infected by eating raw or undercooked fish or squid harbouring infective stages of the nematodes (L3) (Eiras *et al.*, 2016).

5.1.2 Anisakidae occurrence in fish and fish products from European fishery industry

Some of the most important fish species caught by the European fishing industries are at risk of carrying parasites when put on the market (Levsen *et al.*, 2018). In fish, the majority of Anisakidae larvae are typically seen as whitish to greyish, flat, and tight coils, measuring a few mm across (Levsen *et al.*, 2018). Larvae can migrate from abdominal cavity to the flesh, manily *post mortem*, encysting in the fish flesh becoming very hard to detect by the naked eye since they are often transparent and may have penetrated deeply into the fillets. Moreover, the larval occurrence in terms of their abundance and spatial distribution seems largely to depend on ecosystem structure, fish host species and their respective feeding behaviour. Thus, piscivorous species, such as Atlantic cod (*Gadus morhua*), are usually more heavily infected with anisakid larvae compared to strict plankton feeders such as sardine (*Sardina pilchardus*) and capelin (*Mallotus villosus*) (Mladineo and Poljak, 2014; Cipriani *et al.*, 2016; Levsen *et al.*, 2016).

Results from an epidemiological survey involving the economically most important fish species or stocks originating from fishery activities in European fishing grounds showed variable occurrences of Anisakidae larvae in the considered samples (Levsen *et al.*, 2018). The fish species included in the survey were herring (*Clupea harengus*), sardine, anchovy (*Engraulis encrasicolus*), Atlantic mackerel (*Scomber scombrus*), chub mackerel (*S. colias*) and blue whiting (*Micromesistius poutassou*), European hake (*Merluccius merluccius*), haddock (*Melanogrammus aeglefinus*), Atlantic cod and monkfish (*Lophius piscatorius* and *L. budegassa*). Although extremely differente figures for prevalence, abundance and intensity rates where assessed for the different fish species, all the surveyed fish species had been found positive to the presence of Anisakidae larvae (Levsen *et al.*, 2018). Authors also underlined the existence of a strong association between fish host size and larvae occurrence, with larger fish species being more likely to harbour nematodes' larvae. However, the exceptions from this trend for some species such as Atlantic mackerel and anchovy, stressed the relevance of other factors to be considered for a better comprehension of Anisakidae infection patterns in fish species (Levsen *et al.*, 2018).

5.1.3 Anisakiasis clinical syndromes and epidemiology in humans from European countries

In Europe, anisakid nematodes are the most relevant group of parasites in terms of consumer health risk and product quality, with *Anisakis* and *Pseudoterranova* as the genera of major concern, since several species are considered a threaten for human health

(Mattiucci *et al.*, 2017). The term Anisakiasis refers to the zoonotic disease caused by the accidental ingestion of viable larvae of Anisakidae species, harboured by edible parts of fish or squid. Among the nine nominal species belonging to the genus *Anisakis* (Mattiucci *et al.*, 2014), *Anisakis pegreffii* and *A. simplex* (*sensu stricto*) have been acknowledged as zoonotic pathogens (Mattiucci and D'Amelio, 2014). It was further demonstrated that in the Mediterranean countries *A. pegreffii* may cause gastric, intestinal and gastro-allergic clinical syndromes (Mattiucci *et al.*, 2013; Mladineo *et al.*, 2016), while both *A. simplex* (*s.s.*) and *A. pegreffii* larvae may cause allergic reactions in humans (Aibinu *et al.*, 2019).

Anisakiasis is often associated with acute gastrointestinal symptoms such as abdominal pain, diarrhoea, nausea, and vomiting. Moreover, infected patients' clinical status may range from being asymptomatic to requiring emergency room care (Ivanović *et al.*, 2017). Because the symptoms of anisakiasis are non-specific, the disease is often misdiagnosed. For example, in a single study, over 60% of the cases were diagnosed preoperatively as appendicitis, acute abdomen, gastric cancer or Crohn's disease (Ivanović *et al.*, 2017). The *Anisakis*-associated allergic response has been associated with clinical signs such as urticaria and gastrointestinal response (Ivanović *et al.*, 2017). Not only live larvae but also dead worms can cause allergic reaction, due to the thermostability of several allergens, sometimes with serious consequences including anaphylactic shock (Eiras *et al.*, 2016).

Although international regulations, such as Reg. EU No. 1276/2011, demand deep-freezing for at least 24 hours of any fishery product to be consumed raw or semi-raw, this so-called freezing requirement is not necessarily practiced by private households or local guesthouses and restaurants. Thus, consumption of local or privately prepared dishes based on fresh, only lightly processed fish such as boquerones in Spain and marinated anchovies in Spain and Italy, probably represents a major source of anisakiasis in Europe (Levsen *et al.*, 2018).

The risk associated with the consumption of uncooked fishery products deriving from the most important fish species in EU countries is represented in Figure 19 (Levsen *et al.*, 2018).

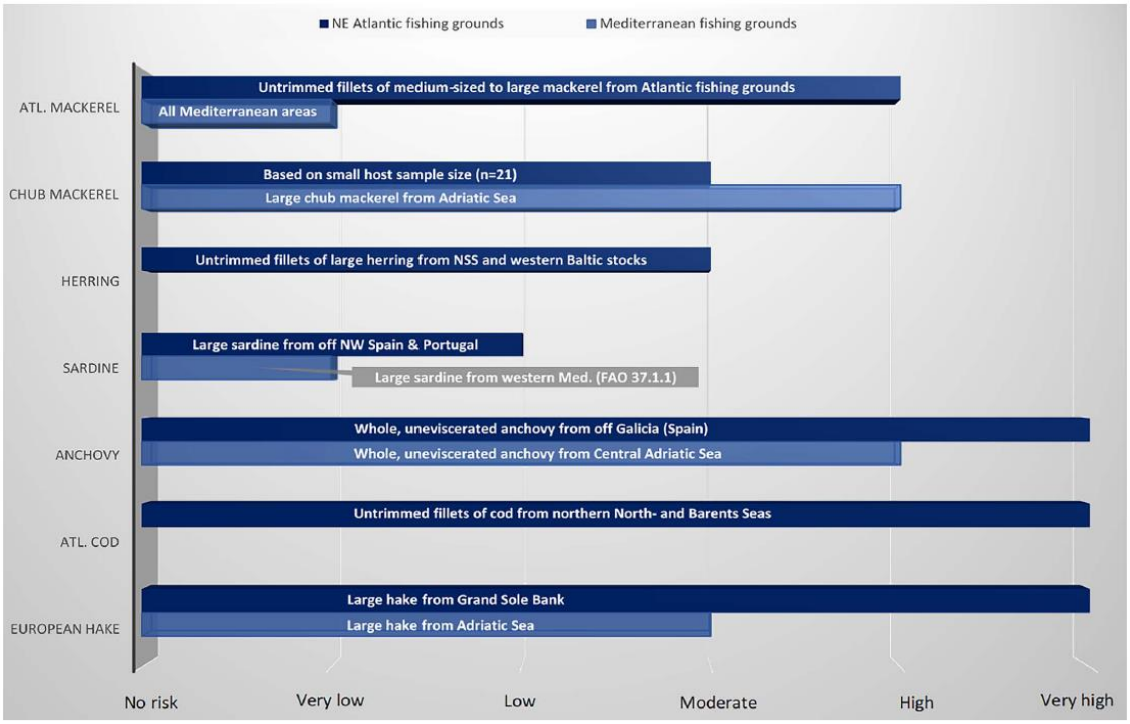

Fig. 19. *Anisakis* spp. exposure risk profile based on parasite prevalence in the flesh of actual fish species if to be consumed raw (sushi/sashimi), or only lightly processed (pickled/marinated/cold-smoked). The risk categories reflect the following *Anisakis* spp. prevalence ranges in the fish flesh: No risk: 0%; Very low: 1–5%; Low: 6–20%; Moderate: 21–50%; High:51–80%; Very high: > 80% (from Levsen *et al.*, 2018).

While anisakiasis occurrence has strongly been related to the consumption of fishery, the risk of becoming infected with *Anisakis* spp. larvae by consuming fish products deriving from European mariculture activities can be considered negligible (Fioravanti *et al.*, 2021). Appropriate feeding procedures in mariculture systems prevent farmed fish from preying parasitized wild species which may enter the sea-cages, thus interrupting the food chain on which Anisakidae life cycle relies on (Fioravanti *et al.*, 2021).

5.1.4 *Eustrongylides* spp.

Nematodes belonging to the genus *Eustrongylides* may affect several fish species and piscivorous birds from freshwater ecosystems and cause the ‘‘big red worm disease’’. The genus *Eustrongylides* includes several species, among which *Eustrongylides tubifex*, *Eustrongylides ignotus*, and *Eustrongylides excisus*, have been related to outbreaks of mortality in nestlings of several orders of birds worldwide (Measures, 1988; Cole, 1999). Nematodes belonging to the genus *Eustrongylides* have an indirect life cycle: the first intermediate host is represented by freshwater oligochaetes, whereas numerous benthic fish can serve as the second host, in which infective larvae (fourth-stage larvae, L4) are encapsulated into muscles and visceral serosae (Paperna, 1974; Cooper *et al.*,1978). Predatory fish, amphibians, and reptiles can be part of the parasite’s cycle as paratenic hosts, accumulating larvae in muscles and/or visceral serosae (Goncharov *et al.*, 2018).

Several species of piscivorous birds act as definitive hosts, among which the great cormorant (*Phalacrocorax carbo*) has often been reported to host adult worms (El-Dakhly *et al.*, 2012; Stocka *et al.*, 2017). In birds, the parasite is most commonly found in the wall of proventriculus, but it can also be harboured in ventriculus and intestine. The worms' penetration in gastro-intestinal walls creates large and tortuous tunnels, with a huge granulomatous reaction around the bodies of dead parasites embedded in host's tissues (Measures, 1988; Cole, 1999).

In the past decades, there has been growing attention on *Eustrongylides* spp. nematodes as zoonotic agents (Eiras *et al.*, 2018). As for other fish-borne parasitic zoonoses, humans become infected by the consumption of raw or undercooked fish harbouring the larval stages of the parasite. Affected humans may develop gastrointestinal syndromes, ranging from mild gastritis or enteritis to stomach and intestinal perforation (Eberhard *et al.*, 1989), and in some cases cutaneous lesions (Eberhard and Ruiz-Tiben, 2014).

Eustrongylides spp. occurrence has been reported worldwide, with different species distribution patterns across the continents. Recently, larval stages of *Eustrongylides* spp. have been reported for the first time in fish from Italian freshwater environments (Dezfuli *et al.*, 2015; Mazzone *et al.*, 2019; Menconi *et al.*, 2020; Guardone *et al.*, 2021). Even though human cases of *Eustrongylides* have never been reported in Europe, the presence of these in fish may represent a threaten for Public Health, since an intense fishing activity exists in Italian freshwater environments.

5.2 Fish-borne zoonotic trematodes

The last WHO report on foodborne trematodes occurrence reported 40 million people to be affected by foodborne flukes' infections (WHO, 2011). The zoonotic trematodes species most commonly associated with the consumption of raw or undercooked fish products belong to the families Heterophyidae and Opisthorchiidae (Chai *et al.*, 2005; WHO, 2011; Chai and Jung, 2017).

Heterophyidae is a family of trematodes affecting vertebrate animals, including mammals and birds (Yamaguti, 1958). More than 30 genera populate this family, among them 13 are known for their zoonotic potential (Chai, 2007). Within this family, *Metagonimus* spp. and *Apophallus* spp. have been reported from human outbreaks in European countries (Caffara *et al.*, 2020).

Opisthorchiidae is a family of trematodes including 33 genera, with zoonotic species reported from human infections in Asia, America and Europe (Waikagul and Thaekham, 2014; Caffara *et al.*, 2020). Three species belonging to 2 genera are considered to be a major zoonotic issue: *Clonorchis sinensis* Cobbold, 1875, *Opistorchis viverrini* Poirier, 1886, and *O. felineus* Rivolta, 1884, among which the last is the only species reported from European countries. Other species belonging to the genus

Metorchis spp., such as *M. bilis* (Braun, 1890), or *Pseudamphistomum truncatum* (Rudolphi, 1819) are considered minor zoonotic pathogens and have been described from Eurasia (Sherrard-Smith *et al.*, 2009; Caffara *et al.*, 2020).

5.2.1 Biology and Life cycle of fish-borne flukes from the families Heterophyidae and Opisthorchiidae

Trematodes belonging to Heterophyidae and Opisthorchiidae families have an indirect life cycle, involving a definitive and two intermediate hosts (Figure 20). Several species of piscivorous mammals and birds can play the role of definitive hosts to these flukes. Adult heterophyids parasitize the small intestine of the definitive hosts, whereas adult flukes from Opisthorchiidae family are usually found in the hepatobiliary system of hosts' liver (Shimazu, 2000; Shumenko *et al.*, 2017; Scaramozzino *et al.*, 2018; Saijuntha *et al.*, 2019; Suwannatrai *et al.*, 2020). Adults release fully embryonated eggs harbouring an already developed miracidium, and eggs are shed in aquatic environments with host faeces.

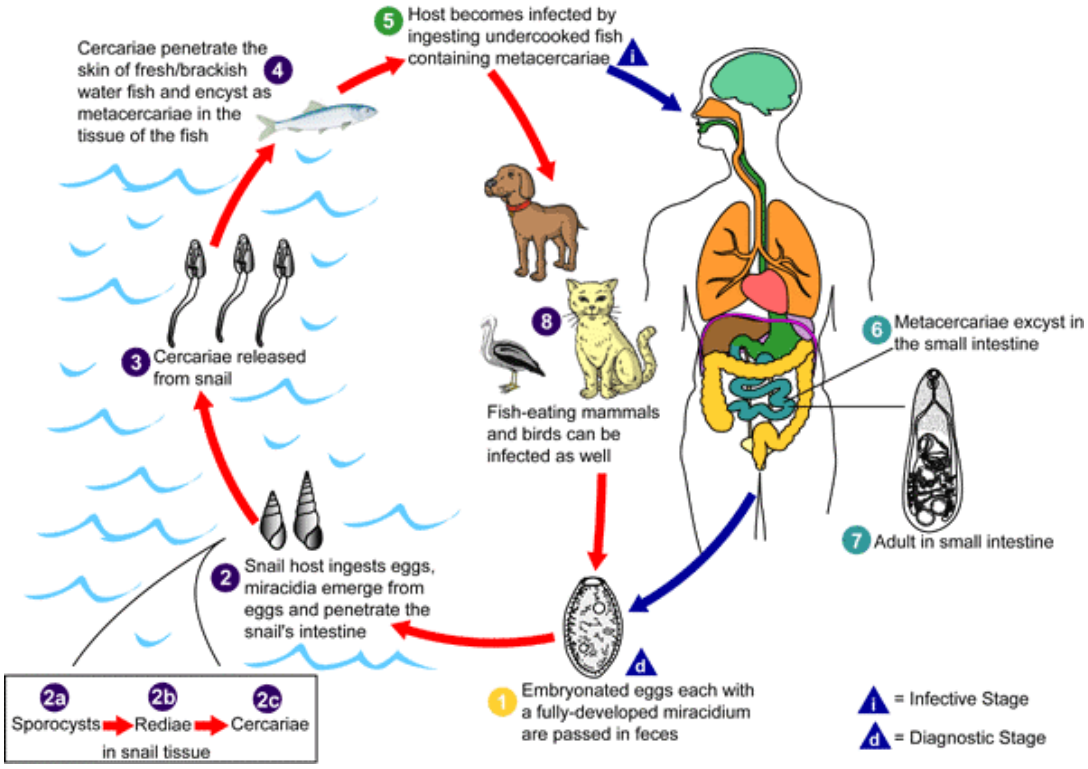

Fig. 20. Heterophyidae and Opisthorichiidae life cycle
https://www.cdc.gov/dpdx/metagonimiasis/index.html

A variety of gastropods are suitable for the role of first intermediate host, mainly depending both on trematode taxonomy and on the biodiversity of the environment. After being ingested by the first intermediate host, eggs hatch and release miracidia, which penetrate the snail intestine. Miracidia undergo asexual reproduction and different developmental phases in the snail up to the production fo several cercariae. Free-swimming cercariae are released from the snail and actively penetrate the second

intermediate host, represented by several species of freshwater and brackish fish, where they encyst as metacercariae, the trematodes' infective stage. Definitive host gets infected by ingesting the fish containing the metacercariae. After being ingested by a suitable definitive host, metacercariae excyst, attach to small intestine mucosa or migrate to the hepatobiliary system, and in these organs, they mature into adults (Shimazu, 2000; Shumenko *et al.*, 2017; Scaramozzino *et al.*, 2018; Saijuntha *et al.*, 2019; Suwannatrai *et al.*, 2020).

As concerns human transmission of fish-borne trematodes, humans get infected through the consumption of raw or undercooked fish or fish product harbouring the infective stages of the parasite, the metacercariae (Chai *et al.*, 2017; Scaramozzino *et al.*, 2018).

5.2.2 Trematodiasis clinical syndromes and epidemiology in humans from European countries

Infections caused by intestinal flukes within the family Hetheropyidae are usually associated with mild and transient clinical pictures, characterised by abdominal pain, diarrhoea, lethargy and weight loss (Chai *et al.*, 2017). However, the severity of clinical manifestation can dramatically increase in highly infected or immunocompromised patients (Chai *et al.*, 2017).

Human infections caused by liver flukes within the family Opisthorchiidae are associated with highly variable clinical pictures. They are usually associated with mild clinical signs including abdominal pain, fever and weight loss (Chai *et al.*, 2005; Pozio *et al.*, 2013). However, chronic opisthorchiasis may cause injuries to the common bile duct, eventually leading to cholangitis, choledocholithiasis, pancreatitis and cholangiocarcinomatosis (Chai *et al.*, 2005; Fürst *et al.*, 2012; Myint *et al.*, 2020).

The highest prevalence of *C. sinensis* and *O. viverrini* infections are in South-East and East Asia (EFSA, 2010), but outbreaks of fish-borne trematodiasis due to *O. felineus* have been reported from European countries, namely Italy, Germany, Lithuania, Poland, Romania, Spain, with high prevalence and hyperendemic areas in the Russian Federation (Sàndor *et al.*, 2020).

As regarding the Italian epidemiologic situation, the first recorded human infections by *O. felineus* in Italy date back to 2003 when a foodborne outbreak occurred (Crotti *et al.*, 2004). From 2003 to 2011 eight outbreaks and four sporadic cases were reported, with a total of 211 confirmed infections. All of the infections had been related to the consumption of raw tench (*Tinca tinca*) caught in the Bolsena and Bracciano Lakes (Pozio *et al.*, 2013).

Recent outbreaks of fish-borne trematodiases have been related in changings in human population behaviours such as migration and novel eating habits (Petney *et al.*,

2013; Scaramozzino *et al.*, 2018). As for other foodborne parasitic diseases in humans, the emergence of both liver and intestinal fluke zoonoses seems to be strongly related to the increasing consumption of raw or partially cooked food and to the chance of transporting perishable fish products wider and easier than before (Broglia and Kapel, 2011).

Despite the large number of intestinal and liver fish borne trematodiases, the spread of these infections through infected fish seems to be relatively restricted, presumably because the majority of the fishes that could transmit these infections are consumed locally (Robertson *et al.*, 2013). However human infections related to the import of infected fish products have already been documented (Yossepowitch *et al.*, 2004; Morsy and Al-Mathal, 2011), thus the potential for enabling the establishment of such parasites in new countries should not be neglected, although that is dependent on environmental contamination with parasite eggs in the faeces and the availability of susceptible intermediate hosts (Robertson *et al.*, 2013)

5.3 Research activities within the field of fish-borne zoonotic parasites diagnosis

Because the modes of human infection are so similar, collectively these zoonoses may in many locations have a much greater aggregate effect than some other better-known parasitic diseases. The difficulties of diagnosis, the complexities of human cultural behaviours and the poor understanding of potential economic costs have made this field simultaneously daunting, scientifically obscure and, therefore, somewhat unattractive to investigators especially in developed countries (Chai *et al.*, 2005).

Implementing the existent diagnostic techniques towards more efficient tools for detecting zoonotic parasites and for understanding their epidemiology in European countries are major topics of joint research activities promoted by EU institutions. Some of the efforts made in this field are reported in the following scientific papers.

The first work reported in this section aimed at assessing a novel diagnostic technique matching morphological and molecular characterisation for the identification of *Eustrongylides excisus* larvae in freshwater fish products. The developed diagnostic tool was further applied to the study of *E. excisus* epidemiology in Central Italy lakes, in light of its recent appearance in these aquatic enviroments for the first time in Italy (Mazzone *et al.*, 2019b).

The second part of the scientific work reported in this section aimed at developing an innovative molecular-based diagnosis assay for detecting metacercariae of trematodes belonging to families Opisthorchiidae and Heterophyidae within freshwater fish products. Due to the small size of the metacercariae, the infective stages for human, these parasites cannot be detected visually in fish and monitoring requires expert application of time-consuming techniques. The aim of this work was to develop a rapid and affordable molecular method based on multiplex PCR for simultaneous identification of

metacercariae of the most common European opisthorchiids and heterophyids in fish or fish products (Caffara *et al.*, 2020).

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SCIENTIFIC CONTRIBUTION

Scientific contribution purposes.

As previously stated in the introductive section, the scientific work herein reported aimed at:

- Reviewing the available diagnostic techniques for the detection of *E. nucleophila* and *C. molnari*, to highlight fields of application and to point out the major deficiencies of each method. This part of the work is reported in Chapter 5.
- Developing novel diagnostic techniques for detecting parasites' presence in host tissues. This part of the work is reported in Chapter 6.
- Studying the distribution of *E. nucleophila* and *C. molnari* within gilthead sea bream Mediterranean aquaculture and at individuating the major risk factors for the detection of the parasites within different cultural systems. This part of the work is reported in Chapter 7.
- Developing novel molecular diagnostic assays for the detection and genetic characterisation of zoonotic parasites from freshwater fish. This part of the work is reported in Chapters 8 and 9.

New achievements in the detection, occurrence, and risk factors individuation of the emerging enteric parasites *Enterospora nucleophila* and *Cryptosporidium molnari* within gilthead sea bream Mediterranean aquaculture.

5. Detecting *Enterospora nucleophila* and *Cryptosporidium molnari* in gilthead sea bream samples: pros and cons of the available diagnostic techniques.

5.1 Diagnostic protocols for the detection of *Enterospora nucleophila*

5.1.1 Fresh smears

Parasitic elements of enteric pathogens are often detected within fresh smears of both intestinal and gastric mucosa scrapings or within stool samples. However, detecting microsporidia from wet tissues smears can be particularly challenging, due to the minute size of these pathogens and their sparsity in host tissue. Thus, microscopic identification of low intensity infections is often overlooked in fresh smears preparations.

Despite that, when high intensity infections occur microsporidians spores may be observed even in fresh smears. This particularly apply to *Enterospora nucleophila*, whose spores has been detected in relatively large aggregates from fresh smears of posterior intestine mucosa scrapings from heavily parasitized fish (Figure 21) (Palenzuela *et al.*, 2014).

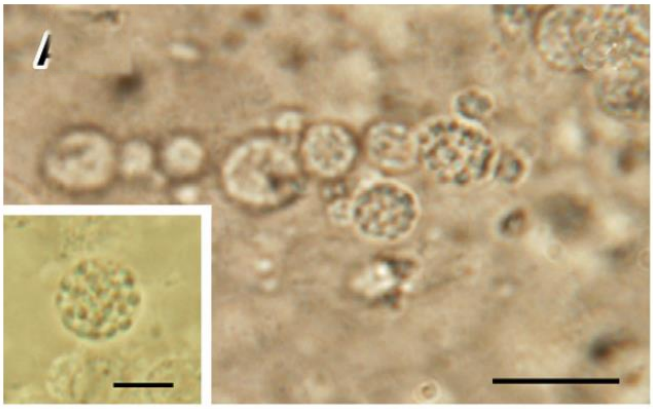

Fig. 21. Fresh smear of posterior portion intestine mucosa of gilthead sea bream showing the presence of *E. nucleophila* spores aggregates (from Palenzuela *et al.*, 2014).

5.1.2 Histology

Until recent years microsporidia had been classified within the Protozoa, but the latest molecular phylogenetic analysis demonstrated a close relationship with Fungi and it is now under questioning that they can be whether a basal branch of the Fungi or as a sister group (James *et al.*, 2006; Han and Weiss, 2017). This close phylogenetic relationship results in a variety of common biological features, such as the presence of chitin in spores' wall, the use of trehalose as the main sugar reserve or some similarities occurring during mitosis and meiosis (Ahmed *et al.*, 2019). These biological traits can be used for diagnostic purpose.

Microsporidians in histologic sections are generally diagnosed by observing spores within host tissues preparations, such as histological slides and tissue/faecal smears.

Spores can be efficiently detected in Haematoxylin and Eosin (H&E) stained sections when they constitute large aggregates or xenomas. In spite of that, individual spores are not frequently detected in host tissues with conventional H&E staining, particularly if spores are scattered within the tissues, areas of inflammation, or small spores in nuclei (Peterson *et al.*, 2011), as for *E. nucleophila* detection.

As for other microsporidians, *E. nucleophila* diagnosis is extremely challenging due to its small dimensions (spore width is about 1.5 µm) and its location within the host cells, since developmental stages can be found either in the cytoplasm and into the nucleus. The histological stains used so far to highlight *E. nucleophila*'s presence in the host tissues are described below. The stage or the stages of the parasite (developmental stages or spores) which can be observed using the considered staining method will be highlighted.

5.1.2.1 Routine staining methods: Giemsa stain and Toluidine blue (TB)

One of the major issues in using routine H&E stain for detecting microsporidia is that spores can be obscured against a similarly staining background, retaining Haematoxylin stain or may not stain at all. Thus, other routinely histological stains had been used in previous works for detecting *E. nucleophila* in gilthead sea bream (*Sparus aurata*) tissues' sections. Observation of Toluidine Blue (TB) or Giemsa-stained histological sections revealed the presence of developmental stages within the nuclei of infected host cells (Figures 22 and 23). In particular, Giemsa led the differential staining of plasmodial and sporogonial stages in intranuclear infections of Rodlet cells (Palenzuela *et al.*, 2014; Picard-Sanchez *et al.*, 2020).

Concerning Giemsa and Toluidine blue stains, they appeared to be effective in staining the parasite's developmental stages in both intracytoplasmic and intranuclear location, as well as spores (Figure 23). However, the lack of distinctive morphological features and the small size of the spores make them easily mis-identified with other elements, such as phagocytosed cell debris, granules of degenerated cytoplasm or aggregates of chromatin.

An experienced microscopist could be able to use the size and shape of the spores to differentiate *E. nucleophila* from other structures, but this procedure would be extremely time-consuming due to the lengthy microscopic examination to recognise the spores, especially if present in low numbers. For these reasons, these staining methods do not apply to routinely diagnostic procedures and can hardly ever be used for diagnostic purpose.

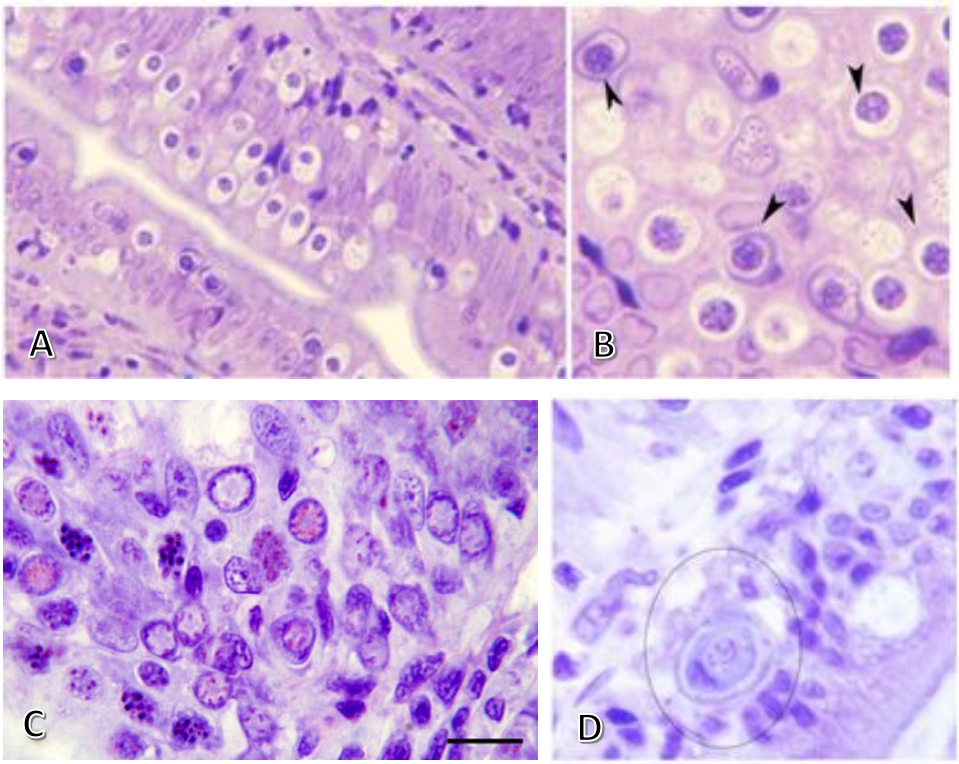

Fig. 22. Giemsa stain. Intestinal mucosa contains abundant infected rodlet cells harbouring intranuclear stages of *E. nucleophila* (insets A and B) (from Picard-Sanchez *et al.*, 2020). Differential staining of plasmodial and sporogonial stages in intranuclear infections (inset C) (from Palenzuela *et al.*, 2014). Intestinal mucosa with a macrophage engulfing an infected rodlet cell (encircled image) (from Picard-Sanchez *et al.*, 2020).

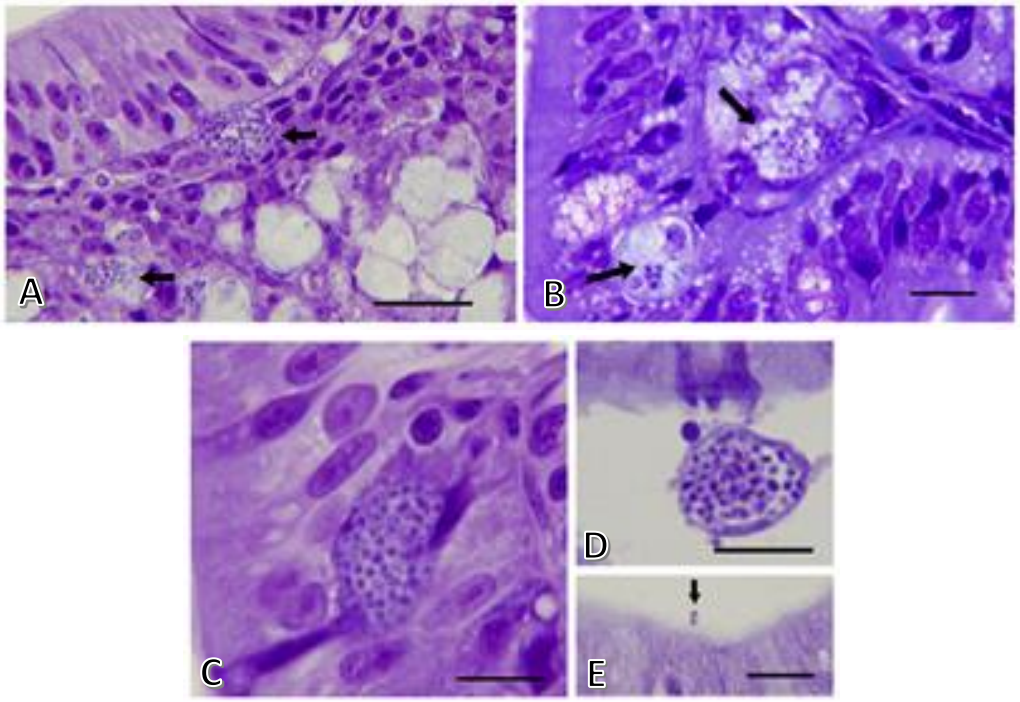

Fig. 23. Toluidine blue stain reveals the presence of *E. nucleophila* within intestinal sections of affected gilthead sea bream. Inset A: spores in a cytoplasmic infection in the submucosa (arrows). Inset B: vacuolated cells and macrophages with cell debris and spores in the epithelium and submucosa (arrows). Inset C: cytoplasmic infection in an enteroendocrine cell. Inset D: infected cell detached from the lumen. Inset E: spore attached to the brush border of an enterocyte (arrow) (from Palenzuela *et al.*, 2014).

5.1.2.2 Fluorescent staining methods: Calcofluor White (CFW) and Uvitex 2B

The fluorochromes Calcofluor White and Uvitex 2B and fluoresce microscopy have been extensively utilized to study fungi structures and host-pathogen interactions.

Calcofluor White (CFW) is a disodium salt of 4,4'-bis-(4anilino-bis-diethyl-amino-S-tri-azin-2-ylamino)-2,2'-stilbene-disulfonic acid, a colourless dye originally used in the textile and paper industries as a whitening agent. CFW binds to β 1–3, β 1–4 polysaccharides in cellulose and chitin and emits fluorescence at a wavelength of 395-415 nm, but it is not specific to microsporidia spores, neither an indicator of viability. CFW can be used to detect many yeasts and pathogenic fungi, including Microsporidia, and is suitable for all types of samples, including fresh, fixed, frozen, and paraffin-embedded tissues (Zhao *et al.*, 2020).

Uvitex 2B is another nonspecific fluorochrome which has been initially used for detecting pathogenic fungi and algae within the host tissues for more than thirty years (Koch and Pimsler, 1987). Uvitex 2B application to microsporidia diagnosis started in the 1990's, when it was used for detecting *Encephalitozoon bieneusi* from HIV positive patients' stool samples (Gool *et al.*, 1993). As for CFW, Uvitex 2B ability to detect the presence of microsporidia within different kinds of samples is related to the capacity of binding chitin molecules and emits fluorescence at a wavelength of 355-425 nm.

In CFW and Uvitex 2B stained sections, numerous fluorescent-stained infected cells were detectable at low magnification and spores were detected in two different cellular locations, the nucleus and/or the cytoplasm (CFW stained sections are not showed in the work) (Palenzuela *et al.*, 2014). Since chitin is a protein of the spores' capsule, spore is the only parasitic stage which can be highlighted using these two techniques.

The use of fluorescent brighteners binding to chitin for the detection of microsporidians largely improves the chances of highlighting microsporidians presence, despite it only facilitates the detection of spores which is the only stage stained (Figure 24) (Picard-Sánchez *et al.*, 2020).

Major difficulties encountered in the interpretation of fluorescent stains include distinguishing microsporidial spores from background, artifactual staining, and determining the precise location of spores within both the tissue sections and host cell itself (Peterson *et al.*, 2011). Despite an increased sensitivity compared to traditional histological stains, the potential for cross reactivity with Fungi still exists, especially in stool specimens. Thus, it has been recommended that

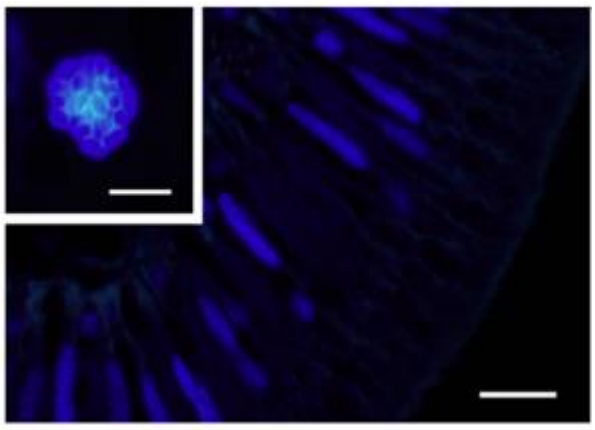

Fig. 24. Infected nuclei stained with the fluorescent brightener Uvitex-2B. Inset: detail of a group of spores. (from Palenzuela *et al.*, 2014).

chemofluorescent brighteners should be used in combination with traditional histological stains, to provide better sensitivity and specificity. However, even the best possible tissue preparation and staining for light microscopy rarely enables a microsporidian species-specific diagnosis (Ghosh and Weiss, 2009).

5.1.2.3 Selective stains: Luna's stain (Luna, 1968)

The Luna's stain was originally developed to detect cytoplasmic granules within eosinophils, Negri bodies, erythrocytes, and phagocytes (Luna, 1968; Tomasi *et al.*, 2008). It also has been used to highlight elastin in tissue sections (Kligman, 1981). A study regarding Luna's stain application to fish microsporidia infections diagnosis showed high sensitivity in detecting the spores within host tissues, with the spores displaying exceptionally high contrast from the background stain with minimal to no interference from the few positively staining tissues elements. This study also showed the ability to detect spores of *Nucleospora salmonis* within the cells nuclei (Peterson *et al.*, 2011).

As concerns *E. nucleophila*, histological sections stained with Luna's stain showed brick red stained spores, with minimal background interference (Palenzuela *et al.*, 2014) (Figure 25).

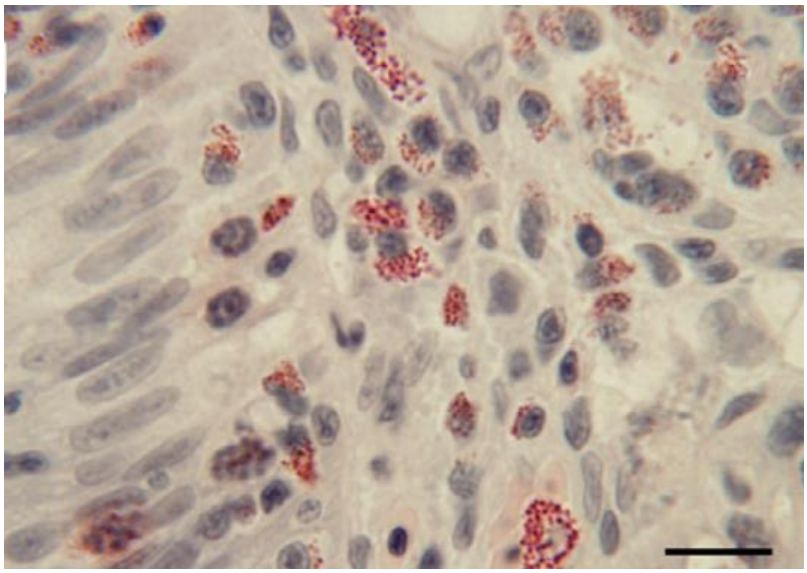

Fig. 25. Mature spores were PAS-positive and stained red with Luna's stain. Granules of eosinophilic granulocytes also stained red (from Palenzuela *et al.*, 2014).

When tissue sections were stained with Luna's stain, PAS-positive mature spores stain brick red, standing out from the background. However, the presence of eosinophilic granulocytes and/or erythrocytes makes the histological picture difficult to understand and may lead to misinterpretations. The granules of these granulocytes and erythrocytes have dye-affinity and dimensions comparable to *E. nucleophila* mature spores. Furthermore, they are not infrequently observed within inflammation pictures, since flogosis is accompanied with hyperaemia in the early stages and eosinophilic

granulocytes are among the most common leukocytes in inflammatory reactions to parasites.

5.1.3 Transmission electron microscopy

Transmission electron microscopy (TEM) is still the gold standard technique for detecting microsporidia at any stages and it is necessary for the definitive identification of the microsporidian species (Palenzuela *et al.*, 2014). The description of polar filament unique structure is required for assigning the parasite to a given species and it can be achieved only by the use of TEM (Ghosh and Weiss, 2009). Species identification is achieved by examining the morphology of ultrastructural organs such as the polar filaments, which are characteristic of each microsporidian species. Ultrastructural features of both developmental stages and spores of *E. nucleophila* and their relationship with host's cell have been described by Palenzuela *et al.* (2014) and are shown in Figure 26.

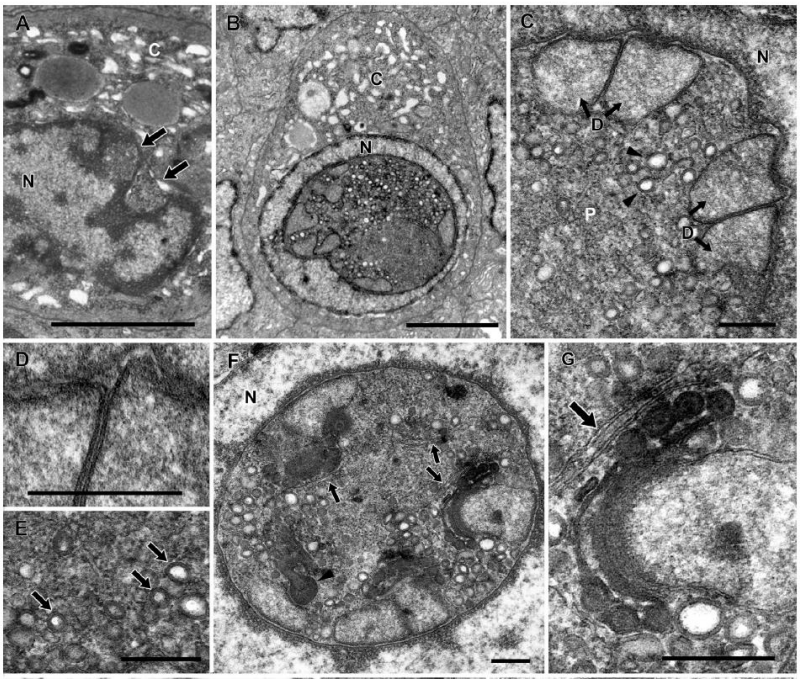

Fig. 26. Transmission electron micrographs showing the ultrastructure of intranuclear stages of *E. nucleophila* in rodlet cells of the intestine of gilthead sea bream (from Palenzuela *et al.*, 2014).

While TEM evidence of the polar filament or other ultrastructural features unique to the phylum is considered incontrovertible proof of microsporidiosis, a more specific diagnosis is not always possible on the basis of morphology alone. Especially in the case of closely related species, distinguishing characteristics may arise in only certain developmental stages of the organism, all of which may not be present in a particular clinical sample (Ghosh and Weiss, 2009). Moreover, TEM application is expensive, time-consuming and not feasible for routine diagnosis (Polley *et al.*, 2011).

5.1.4 Molecular Analysis

In order to apply PCR-based diagnostics to a pathogen, some genetic sequence information must be known in advance. Pathogen microsporidia are an extremely diverse group of emerging pathogens, and the available genetic information on these organisms is limited, especially for microsporidia affecting aquatic organisms, but ever increasing (Ghosh and Weiss, 2009). For the majority of the microsporidia molecularly characterized so far, rRNA genes sequences on GenBank are the only genetic information available. Thus, due to the availability of sequence information as well as the presence of conserved and variable regions within the rRNA genes, PCR-based methods have typically utilized primers complementary to small sub unit (SSU) rRNA gene for the characterization of the microsporidia (Ghosh and Weiss, 2009).

Diagnostic studies using primers to the various rRNA genes of microsporidia have been reviewed by Weiss and Vossbrinck (1999) and Franzen and Muller (1999). Some of the reported primers are species-specific whereas the majority of them are generic primer sets that amplify several genera. Thus, incontrovertible species-specific diagnoses must rely on downstream restriction analysis or on amplicons sequence analyses.

5.1.4.1 PCR assay for *E. nucleophila*

The traditional PCR assay for the detection of *E. nucleophila* employs DNA extracted from fish intestinal mucosa scrapings and taxon-specific primers ss18f and ss1492r targeting the SSU rRNA gene (Ghosh and Weiss, 2009). These primers bind to a highly conserved region of the SSU rDNA and lead to the amplification of a 1,298 bp DNA product. As for other microsporidians, the definitive species-specific diagnosis must be achieved through PCR product sequencing.

5.1.4.2 Real Time PCR assays

A number of probe-based real-time PCRs has been developed for the detection of different microsporidian species within a sample (Wolk *et al.*, 2002; Menotti *et al.*, 2003), whilst a multiplex real-time PCR has been developed for the detection of human relevant pathogens, such as of *Enterocytozoon bieneusi* and *Encephalitozoon species* (Verweij *et al.*, 2007).

As highlighted by Polley *et al.* (2011) SYBR Green PCR specificity relies on the downstream analysis of amplicons melting curves, which allows to exclude the amplification of non-specific products or dimers originating from the incorrect pairing of the probes. Thus, SYBR Green-based assays must lead to the amplification of qPCR products with clearly different melting temperatures for discriminating among different genera or species which may be present in a sample at the same time.

Since most of the existent Real Time PCR assays amplify the SSU rRNA gene, which is highly conserved among microsporidians, amplicons from different species or

even genera may have similar melting temperatures. Therefore, further sequencing of the qPCR product may be required for a definitive species-specific diagnosis.

For routine diagnostics, an assay should be easily performed (thus eliminating the possibility of errors during processing), affordable and it must show good sensitivity and specificity. Conversely to traditional PCR assay, Real-time PCR analyses remove the requirement for the separate amplification and analysis of amplicons, providing a rapid, sealed-tube system. This format decreases both the turnaround time and the possibility of contamination of subsequent reactions by amplicons released into the environment during gel loading. Thus, real-time PCR offers considerable advantages for routine diagnosis or disease surveillance plans, tempered only by the initial cost of the instrument required (Polley *et al.*, 2011). SYBR Green PCR assays low specificity in differentiating closely related genera or species may be improved by the use of TaqMan probes, which eliminates non-specific fluorescence phenomena.

A SYBR Green-based assay which employs oligonucleotide probes targeting the SSU rDNA gene of *E. nucleophila* has been recently published by Picard-Sánchez *et al.* (2020). Since this technique has been widely employed in the experimental work of our laboratory, the methods will be later described.

5.2 Diagnostic protocols for the detection of *Cryptosporidium molnari*

Cryptosporidium spp. are responsible for outbreaks of waterborne and foodborne diseases worldwide and it has been estimated that human foodborne cryptosporidioses have an incidence of 8 million cases per year globally (Ryan *et al.*, 2018). Due to concern on *Cryptosporidium* spp. as a Public Health threaten, there have been many efforts to develop increasingly sensitive and specific diagnostic techniques.

For this reason, histological assays have given way to molecular diagnostic techniques for the detection of *Cryptosporidium* spp., which are less time-consuming and far more suitable for routine diagnostic. Even though histology and electron microscopy are still considered the gold standard for understanding the pathological effect of the parasite and to study the morphology of taxonomic-useful features (Zahedi and Ryan, 2020). Hence, they are still widely used. The histological stains used so far to highlight *Cryptosporidium molnari* presence in the host tissues are shown below. Molecular assays will follow.

5.2.1 Tissue and faecal smears

Fresh sample examination is mainly used for detecting *Cryptosporidium* spp. from raw food, water (Kaupke *et al.*, 2019) or faecal samples (Chartier *et al.*, 2013). In order to increase the sensitivity of this techniques, oocysts within samples are often concentrated before microscopic examination (Kaupke *et al.*, 2019). In order to achieve higher specificities and sensitivities, fluorescently labelled monoclonal antibodies directed against the oocyst wall or rapid stains may be used.

As concerns *C. molnari* detection, stomachal mucosa scrapings and faeces samples smears from parasitized gilthead sea bream have been examined. Light microscopy was performed on fresh, Giemsa, Toluidine Blue (TB) and Kynioun acid-fast stained specimens. Oocysts have been detected in fresh, Giemsa stained and TB stained smears of faecal and mucosal scrapings samples (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla and Alvarez-Pellitero, 2003; Sitjà-Bobadilla *et al.*, 2005; Palenzuela *et al.*, 2010). However, the assignment of oocysts detected by light microscopy to a precise species has to be performed with the use of TEM or molecular tools.

5.2.2 Histology

Both traditional detection methods (consisting in light and electron microscopy, antibody, and enzyme-based assays) and molecular tools are currently used for detection of *Cryptosporidium* and each one has its peculiar sensitivity and specificity. Traditional diagnostic techniques, especially light microscopy, are still commonly used because they are relatively simple and cost efficient (Figure 27). Despite this, traditional methods showed lack sensitivity and specificity, making them not always reliable (Zahedi and Ryan, 2020).

Immunological assays based on either antigen or antibody detection can offer higher specificity and sensitivity than traditional microscopy and thus they are widely used. However, most of the antibody and enzyme-based assays which are routinely used for the detection of human and terrestrial animals *Cryptosporidium* spp. (Fayer and Xiao, 2007) have never been employed for the detection of this protozoan in fish samples.

In order to highlight the presence of *C. molnari* within fish tissues and the related histological damage, thin sections of small pieces of stomach have been analysed (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla and Alvarez-Pellitero, 2003; Sitjà-Bobadilla *et al.*, 2005).

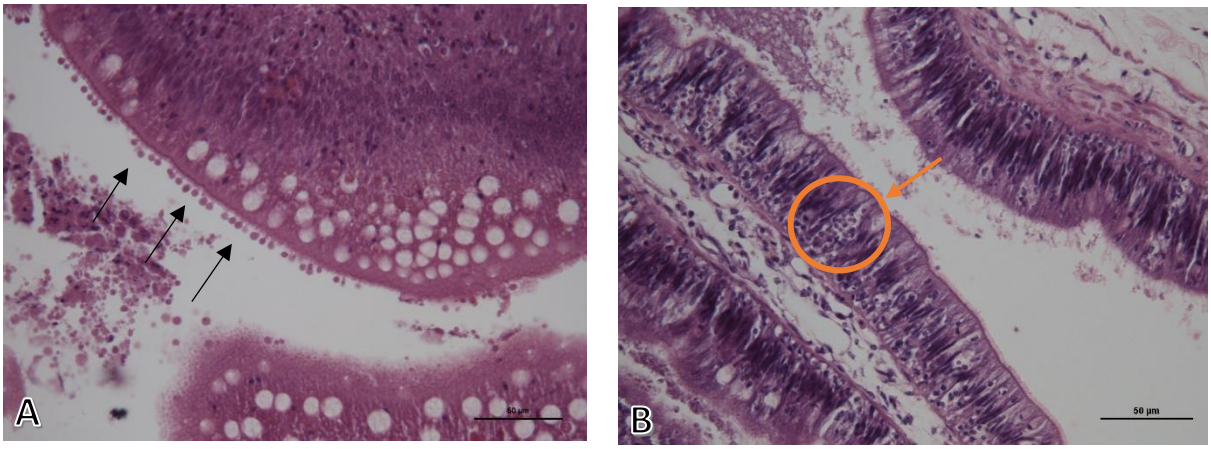

Fig 27. Intestine histological section of turbots (*Scophthalmus maximus*) infected by *C. scophtalmi*. Inset A: multiple round to oval extracytoplasmic stages of *Cryptosporidium* spp. adhering to the luminal surface of the epithelial cells (black arrows). Inset B: intra-epithelial sporogonic stages located deeply within the enterocytes layer (arrow). Pictures were taken at Fish pathology laboratory- DIMEVET.

In histological sections, oocysts measuring 4-5 µm can be detected deeply within the epithelium, with a distinct colourless wall. Four naked, curved, vermiform sporozoites can be observed within each oocyst. Histopathological damage due to the multiplication of the parasite within the stomachal mucosa can also be observed. Necrotic area, vacuolation, and sloughing of epithelial cells are clearly visible in the affected tissues (Alvarez-Pellitero and Sitjà-Bobadilla, 2002). In fish which have passed the infection it is not possible to detect any parasitic stage. However, in these fish stomach epithelial layer shows massive vacuolation and abundant debris appeared within the cells and in the lumen. In spite of the destructive effect, no evident inflammatory is usually visible, except for occasional clusters of rodlet cells (Alvarez-Pellitero and Sitjà-Bobadilla, 2002).

Despite a good sensitivity, histological techniques are unable to provide any information on the species or genotype of *Cryptosporidium* detected in the tested samples. Since species identification and genotyping are fundamental for epidemiological surveys or for determining whether a *Cryptosporidium* species may have zoonotic potential, molecular techniques are widely used (Xiao and Feng, 2017).

5.2.3 Transmission electron microscopy

Transmission electron microscopy (TEM) is the most suitable technique for detecting *Cryptosporidium* spp. within the host tissues and for studying their morphology. It is particularly useful in studying ultrastructural features which are used for protozoan taxonomy, such as sporozoites and oocysts morphology and dimension.

The ultrastructure of *C. molnari* has been studied exclusively from infected stomachs of gilthead sea bream. All stages were located within a host-contributed parasitophorous vacuole, initially formed at the microvillous surface of epithelial cells. Merogonial and gamogonial stages has been observed mainly in this extra-cytoplasmic position, whereas oogonial and sporogonial stages has only been observed deeply in the epithelium, as in other piscine *Cryptosporidium* spp.. It has been also possible to notice absence of sporocysts and flagelles in microgametes, and the presence of four sporozoites (Alvarez-Pellitero and Sitjà-Bobadilla, 2002).

5.2.4 Molecular Analysis

Immunological assays, based on either antigen or antibody detection can offer higher specificity and sensitivity and are widely used, however, some commercially available immunochromatographic assays have low sensitivity and specificity.

Molecular tools are increasingly being used for genotyping and subtyping of *Cryptosporidium* spp. originating from farm, domestic and wild animals, and for monitoring the sources of infections and transmission routes. As traditional techniques are unable to provide any information on the species or genotype of Cryptosporidium

present in samples, molecular techniques are widely used (Xiao and Feng, 2017; Gerace *et al.*, 2019).

The most commonly used locus for detecting *Cryptosporidium* is the 18S rRNA gene, as it is conserved enough to be able to detect all species of Cryptosporidium but also contains a hypervariable region for differentiating species and genotypes (Xiao and Feng, 2017). Quantitative PCR assays are increasingly being used for species specific detection and to quantitate parasite loads (Xiao and Feng, 2017) for both research and clinical purpose.

Currently, most *Cryptosporidium* genotyping tools use PCR targeting the small subunit (SSU) rRNA gene, largely because of the existence of conserved *Cryptosporidium* specific sequence for designing primers that allow broad specific detection of all *Cryptosporidium* spp. and semi-conserved and hypervariable regions that can be used for the differentiation of various species and genotypes by restriction fragment length polymorphism (RFLP), melting curve, or DNA sequence analyses (Xiao, 2010).

Among the SSU rRNA-based *Cryptosporidium* genotyping tools, the PCR-RFLP using nested PCR amplification of a ~830-bp fragment and restriction analysis of the secondary PCR products using enzymes SspI and VspI is the most commonly used one (Xiao and Feng, 2017). It can be used in genotyping *Cryptosporidium* spp. from both humans and animals.

PCR tools targeting other genes were used in early *Cryptosporidium* research, but they are now infrequently used in genotyping because of their narrow detection range. They can only be used for genotyping *Cryptosporidium* species that are closely related to *Cryptosporidium parvum* or *Cryptosporidium hominis* because of the nature of sequences used in primer design (Xiao and Feng, 2017).

Thus, tools based on the amplification of the oocyst wall protein (COWP) have limited usefulness in genotyping *Cryptosporidium* spp. from animals since they were originally designed for amplifying and differentiating *Cryptosporidium meleagridis*, *C. parvum, C. hominis* and species/genotypes closely related to *C. parvum*.

Despite a good specificity when employed for genotyping human *Cryptosporidium* spp., these assays led to the identification of *C. parvum* from unexpected animal species, raising doubts about their reliability when used with non-human Cryptosporidia genome (Xiao, 2010). Moreover, subtyping tools have been used extensively only in studies on the transmission of *C. hominis* in humans and *C. parvum* in humans and ruminants. One of the most employed subtyping tools is the DNA sequence analysis of the 60 kDa glycoprotein (gp60, also called gp40/15), which leads to the identification of the major zoonotic subtype related to *C. parvum* and *C. hominis* (Xiao, 2010).

As concerns fish *Cryptosporidium*, three species have been described from piscine hosts and not found in other species to date. These are *Cryptosporidium molnari* (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Palenzuela *et al.*, 2010), *Cryptosporidium*

scophthalmi (Alvarez-Pellitero *et al.*, 2004), and *Cryptosporidium huwi* (formerly piscine genotype 1) (Ryan *et al.*, 2015).

Molecular characterisation has also identified eight piscine genotypes (piscine genotypes 2 to 9) and two different *C. molnari*-like genotypes, which have yet to be assigned to a given species (Certad *et al.*, 2019; Couso-Pérez *et al.*, 2019). Moreover, more than 8 unnamed novel genotypes, *Cryptosporidium xiaoi*, *Cryptosporidium scrofarum*, *C. parvum*, *C. hominis*, and rat genotype III have been detected from fish samples (Certad *et al.*, 2019).

Only three studies have been conducted on gp60 subtyping *Cryptosporidium* DNA isolated from wild fish samples (Reid *et al.*, 2010; Koinari *et al.*, 2013; Certad *et al.*, 2015), although this tool has become relatively standard for identifying subtypes in potential reservoir species for human infection. None of these assays has been employed for genotyping *C. molnari* to date. Thus, further studies aimed to implement molecular tools suitable for discriminating the *Cryptosporidium* species infecting fish and, above all, to identify potentially zoonotic ones must be developed.

5.1.4.1 Nested PCR assay for the detection of *C. molnari*

Molecular diagnosis of *C. molnari* has been performed by the amplification of two different loci within the SSU rDNA and by the amplification of a actin gene fragment (Palenzuela *et al.*, 2010). DNA samples used in these assays had been extracted from small portions of gilthead sea bream stomachal mucosa.

Nested PCR assays targeting the SSU rRNA gene have followed previously reported protocols (Xiao *et al.*, 1999; Fayer *et al.*, 2001; Ryan *et al.*, 2003) and have led to the amplification of a 823 bp fragment and a 572 bp fragment. Nested PCR protocol targeting the actin gene has been carried out according to Sulaiman *et al.* (2002) and has led to the amplification of a 1,103-bp fragment. Species specific diagnosis has to be confirmed by the sequencing of the amplicons (Palenzuela *et al.*, 2010).

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Title: Detection of the Intranuclear Microsporidian *Enterospora nucleophila* in Gilthead Sea Bream by *In situ* Hybridization

Short running title: ISH detection of *E. nucleophila*

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ABSTRACT

Enterospora nucleophila is an intranuclear microsporidian responsible for emaciative microsporidiosis of gilthead sea bream (GSB). Its minute size and cryptic nature make it easily misdiagnosed. An *In situ* hybridization (ISH) technique based on antisense oligonucleotide probes specific for the parasite was developed and used in clinically infected GSB in combination with calcofluor white stain (CW) and other histopathological techniques. The ISH method was found to label very conspicuously the cells containing parasite stages, with the signal concentrating in merogonial and sporogonial plasmodia within the infected cell nuclei. Comparison with CW demonstrated limited ISH signal in cells containing mature spores, that was attributed mostly to the scarcity of probe targets present in these stages. Although spores were detected in other organs of the digestive system as well as in the peripheral blood, proliferative stages or parasite reservoirs were not found in this work outside the intestines. The study demonstrated a frequent disassociation between the presence of abundant spores and the intensity of the infections as determined by the parasite activity. The ISH allows confirmatory diagnosis of GSB microsporidiosis and estimation of infection intensity, and will be a valuable tool for a more precise determination of parasite dissemination pathways and pathogeny mechanisms.

Keywords: Parasite, Microsporidia, Enterospora, In-situ Hybridization, Diagnostics

1 INTRODUCTION

2 Microsporidians are minute intracellular parasites that infect all animal phyla and even
3 protists like amoebae and gregarines (Larsson 2000, Scheid et al. 2008). Over half of
4 the known genera infect aquatic organisms (Stentiford et al. 2016). With a close
5 phylogenetic relationship with fungi, they share biological features such as the
6 presence of chitin in the spores, the use of trehalose as the main sugar reserve or
7 some similarities during mitosis and meiosis (Han and Weiss, 2017). Like in the
8 Rozellomycota, adaption to obligate intracellular parasitism has led to gene loss,
9 genome reduction and dependence upon the host for cell function (Quandt et al,
10 2017, Ndikumana et al., 2017). While many species tend to be considered secondary
11 pathogens or linked to immunocompromised condition in human or veterinary
12 medicine, numerous microsporidioses are involved in serious losses in aquaculture
13 settings (El Alaoui et al, 2006, Kent et al. 2014, Palenzuela et al. 2014, Stentiford et al.
14 2016).
15 Multiple species of microsporidians have been reported in gilthead sea bream, *Sparus*
16 *aurata* (GSB) (Faye et al. 1988, Abela et al. 1996, Athanassopoulou 1998, Morsy et al.
17 2013, Mathieu-Daude et al.) but seldom related to clinical diseases in aquaculture
18 settings. An intranuclear species was found in Spanish GSB farms since the 00s, and its
19 association with emaciative disease and dropping mortality was further established as
20 new cases appeared in different facilities. This parasite was described as *Enterospora*
21 *nucleophila* and its closest known relative is *Enterocytozoon hepatopenaei* (Palenzuela
22 et al. 2014), a serious shrimp pathogen widespread in Asia (Thitamadee et al. 2016,
23 Rajendran et al. 2016) and, recently, in South America (Tang et al. 2018). Since the
24 emergency of the disease, it has been also detected in Italian and Greek GSB farms,

and in different facilities including on-growing net cages and land-based GSB nurseries (Caffara et al. 2014 and authors' unpublished data). However, clinical infections only affect certain fish lots or cages and they are not usually widespread within a given facility. In addition, clinical presentation does not always correlate with detection of microsporidian spores in large quantities and, likewise, presence of the parasite has been registered in the absence of clinical symptoms. While specific PCR tests are an invaluable tool for parasite surveillance and epidemiology, interpretation of the disease dynamics and risk assessment are difficult to approach without deep knowledge of the parasite development and pathogenesis.

The diagnosis of *E. nucleophila* is quite challenging due to its tiny size (spore width is about 1 µm) and relatively sparse intranuclear development in certain intestinal epithelial cells. The detection in histology slides is mainly limited to the spore stage, and even these are difficult to identify in routine slides except when present in large quantities. Use of fluorescent brighteners binding to chitin (e.g. calcofluor white) for the detection of microsporidians largely improves the sensitivity in these contexts (Freeman et al. 2013, Palenzuela et al. 2014, Alarcón et al. 2016, Herrero et al. 2018), yet it only facilitates the detection of spores which is the only stage stained. In situ hybridization techniques have been successfully used to circumvent similar limitations in the closely related microsporidian *Enterocytozoon hepatopenaei* (Tang et al. 2015, Rajendran et al. 2016), or in *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) (Weli et al. 2017). The main objective of this work was to develop an ISHbased technique to detect *E. nucleophila* stages, getting insights into the development of the parasite and the pathogenesis of GSM emaciative microsporidiosis.

MATERIALS AND METHODS

Fish

Sparus aurata specimens were collected from a Spanish GSB offshore net pen farm located at Southern Castellón (Comunidad Valenciana region). The facility had a background of *E. nucleophila* infections detected in previous surveys by our laboratory. During the winter season of 2016, a cage showing clear growth retardation and anorexia, abnormally high mortality rate and size segregation within the stock, which are signs of presumptive *E. nucleophila* infection in GSB (Palenzuela et al., 2004), was sampled. Fish were harvested using nets and the smaller, wasted fish were handpicked by the pathologists. Approximately 250 specimens (average weight: 43.4 g) were transferred alive to the Institute of Aquaculture “Torre de La Sal” (IATS) and maintained in 500L fiberglass tanks supplied with a flow-through natural water supply. The specimens were anaesthetized, sacrificed and sampled following routine procedures in compliance with European (86/609/EEC) and National (Royal Decree RD1201/2005) for the protection of animals used in scientific experiments, and approved by the CSIC ethics committee and IATS Review Board. A total of 60 individual fish were euthanized and sampled for downstream analyses. During the necropsies, small pieces of the anterior, middle and posterior intestine were dissected. Samples from different organs were also collected (liver, spleen, stomach, skeletal muscle, gall bladder, brain, gonads, head kidney and trunk kidney). Tissue pieces were immediately transferred to histology cassettes and fixed in 10% neutral buffered formalin for 1-2 weeks, before embedding into paraffin blocks following routine histological procedures. Some subsamples of tissue were embedded into methacrylate resin

Technovit 7100 (Kultzer, Werheim, Germany).

Histology and calcofluor white staining.

Paraffin sections (5 µm thickness) corresponding to the organs of 60 fish were cut and mounted onto electrostatically charged glass slides (Superfrost-Plus, Thermo Fisher Scientific, Spain). They were stained with 0.1% calcofluor white M2R stain (CW) in ddH2O with a drop of 10% potassium hydroxide, for 1 min. The slides were rinsed immediately with 30% ethanol. A light counterstaining was achieved with 0.1% Evans blue solution containing a few drops of glacial acetic acid for 1 min. Slides were examined using an Olympus BX51 fluorescence microscope under UV excitation light. Plastic sections (1 µm) were routinely stained with toluidine blue and examined under visible light.

E. nucleophila probes design and In Situ Hybridization (ISH) procedure

The small subunit ribosomal DNA sequences of *E. nucleophila* isolates (Palenzuela et al. 2014) were used as target. A dataset containing all the sequences available under the category “microsporidia” (2,450 entries, 209 Enterocytozoonidae) in the SSU_r132 database release by SILVA (Pruesse et al. 2007, www.arb-silva.de) was downloaded. The alignment was pruned to the closest related Enterocytozoonidae sequences and then refined manually according to secondary structure criteria using ARB software (Ludwig 2004). Unique *E. nucleophila* oligonucleotide segments, or segments with sufficient variation with respect to the closest taxa, were visually identified in the alignment. Oligonucleotide antisense probes were designed to hybridize with the positive strand of the gene (i.e., binding to both rDNA and rRNA of the parasite) at these regions. Choice of the best probes according to specificity and thermodynamic properties was aided by the software package OLIGO-7 (Rychlik 2007). Two

oligonucleotides were chosen: Enu_725L25 (5'TCC CAC ACC AA CAC CAC TTT CAT A-3') and Enu_101L25 (5'-ATC CGT TCC GCC ATC TAT GTA CAT C-3'). They were modified with 5' digoxigenin labels and sourced from a commercial supplier (Metabion Int., Germany), suspended at 100 uM in TE, and mixed in equimolar amounts as a concentrated stock.

A panel of 32 samples was chosen representing individuals with assorted infection status as determined by the results of the histopathological examination, including Individuals in which few or no microsporidian spores were detected with CW. The ISH procedure was carried out with these probes as described previously for other fish parasites (Palenzuela & Bartholomew 2002, Cuadrado et al. 2007, Constenla et al. 2016), with some modifications. Deparaffinized and hydrated sections were treated with 15 $\mu g.ml^{-1}$ proteinase K (45 minutes at 37 ^{o}C). They were then washed, denatured, and incubated overnight at 37 ^{o}C with the mixture of probes diluted 1:500 in hybridization buffer. Stringency washes were made twice in each decreasing concentrations of SSC buffer (2x, 1x, and 0.25x), for 20 min each, at 40 ^{o}C under a gentle rocking motion. The slides were equilibrated in 1x Genius Buffer at RT and the immunological detection was conducted with AP-conjugated Fab fragments from AntiDig sheep antiserum (Roche Diagnostics, Manheim, Germany), diluted 1:750 in blocking solution and incubated for 2 hours at RT. The colorimetric reaction was performed with NBT/BCIP substrate (nitro-blue tetrazolium chloride/ 5-bromo 4-chloro- 3'indoly phosphate- p toluidine salt) for 2-8 hours at room temperature, until optimum signal with low background was achieved. The slides were counterstained with light green (1%), mounted in permanent medium and observed by light microscopy.

RESULTS AND DISCUSSION

The histopathological study of clinically infected samples stained with fluorescent brightener calcofluor white M2R clearly showed the localization of *E. nucleophila* spores within the nuclei of enterocytes and rodlet cells (RCs), typically bunched in a berrylike pattern clustering up to 25 spores (Fig. 1A). Spores were also detected in the connective tissue and submucosae, either scattered or in large clusters, the later most often within or around melanomacrophage centers (MMCs) (Fig. 1B). The findings were consistent with previous histopathological and ultrastructural studies of this infection (Palenzuela et al. 2014) demonstrating the primary intranuclear development of the parasite in epithelial cells and its presence in cytoplasmic position within other cells, including phagocytes at subepithelial layers. However, the increased sensitivity of the fluorescent stain facilitated the detection of scattered spores in other locations, as a consequence of degenerative changes in the epithelium, spore detachment, and host cellular immune responses (Palenzuela et al. 2014, Sitja-Bobadilla et al. 2016). Scarce spores were found in stomach, gall bladder, peripheral blood, or liver samples (Figs. 1C, 1D, and data not shown). Calcofluor white M2R is widely used to detect microsporidian species due to the high affinity of this compound to bind the chitin present at the microsporidia endospore membrane (Han and Weiss, 2017). Other chemofluorescent agents, mostly stilbene derivatives typically used as whiteners in detergents and in paper and fabrics manufacture, present similar properties acting as chitin-specific stains (e.g., Hoch et al 2005, Rüchel & Schaffrinski 1999). Their use is considered a credible technique for the diagnosis of microsporidians (Didier et al 1995, García, 2002). However, these stains only bind to spores and not to other stages of microsporidian cells devoid of chitin, and therefore

145 other strategies are needed to study microsporidian cells development in the host
146 (Chen et al, 2017).
147 The ISH procedure produced intense blue-purple signals in clinically infected fish,
148 particularly in the intestinal mucosa (Fig. 1E). Detailed examination and double
149 staining with ISH and CW showed that the bulk of the ISH signal concentrated in cells
150 containing microsporidians under proliferative, pre-sporogony development phase
151 and in which *E. nucleophila* spores were not present (Fig. 1F). Indeed, scarce ISH signal
152 was present in mature spores which appeared mostly unstained when present
153 (Fig.1G). This pattern resulted quite revealing for the confirmation of the infection in
154 samples presenting the typical epithelial hypercellularity and nuclei alterations (Fig.
155 1H) often associated to the *E. nucleophila* infection (Palenzuela et al. 2014), but in
156 which confirmatory diagnosis was not possible due to the absence of spores in routine
157 histopathological examination or in calcofluor-stained sections. The staining was a
158 clear result of the design of antisense probes targeting parasite rRNA present in the
159 infected host cells, most often within the nuclei (Fig 1I). This approach resulted in
160 enhanced sensitivity and, more importantly, in the ability to detect metabolically
161 active parasite stages containing many ribosomes. Light but noticeable ISH
162 background was present in some samples from infected stocks in which neither spores
163 nor clearly ISH-positive cells were present, but presenting large numbers of
164 granulocytes and other histopathological alterations associated to the infection (not
165 shown). Since background signal was never present in control uninfected samples or in
166 control slides without probes, it was interpreted to be caused by parasite remnants
167 present in late infections. However, it was not possible to determine if these patterns

indicated a recovery stage or a covert infection from which the parasite development could be reactivated.

The scarce or absent ISH signal in mature spores is most likely related to a low number of probe targets. Since scarce or null protein synthesis activity is present in mature spores, the signal is mostly limited to parasite genomic rDNA copies. Some microsporidians have extremely condensed genomes and scarce ribosomal gene arrays, like *Encephalitozoon intestinalis* with the smallest known nuclear genome (2.3Mbp) and only 11 rDNA arrays per haploid genome (Corradi et al. 2010). Enterocytozoonidae species closely related to *E. nucleophila* are included in this trend, with recent genome assemblies of 3.1 and 3.26 Mbp in *Enterospora canceri* and *Enterocytozoon hepatopenaei*, respectively (Wiredu Boakye et al. 2017). However, the low signal intensity observed in spores could also be partly related to incomplete permeabilization of their shells. A very similar ISH staining pattern was described with anti-sense RNA probes for the salmon microsporidian *Desmozoon lepeophtherii*, which labelled strongly ribosome-rich merogonial and plasmodial stages (Weli et al. 2017). In our procedure, the use of pre-labelled synthetic oligonucleotide probes instead of longer lab-made RNA probes and has obvious advantages for practical purposes. Even though ISH allowed clear detection of *E. nucleophila* developmental stages in GSB tissues, these were not found in other organs except the intestine. Using CW staining, however, microsporidian spores were occasionally found isolated or in small clusters in peripheral blood, skeletal muscle, gall bladder, liver and stomach in some clinically infected fish. These appear to be related to systemic spreading of advanced infections and parasite clearing mechanisms more than to previously unidentified parasite development niches in the fish. This observation is consistent with the pathogeny of

192 the disease, which is mostly evidenced as a chronic severe emaciation and growth
193 arrestment due to intestinal dysfunction. By ISH, it was evidenced that heavy
194 infections in clinically infected fish are not consistently associated with the presence of
195 large numbers of spores and, likewise, these tend to be present more abundantly in
196 samples with limited overall parasite activity. The limited epidemiological data on
197 emaciative microsporidiosis of GSB points to it as a chronic condition, as it results in
198 size segmentation of infected and uninfected fish within the same cage, reaching
199 levels equivalent to year-class differences in some cases (Palenzuela et al. 2014 and
200 author's unpublished observations). This suggests either a very slow development
201 cycle or an equilibrium between clearing of spores and reinfection of regenerated
202 epithelia. The development in the intestinal mucosa certainly opens the path for a
203 direct release and dispersion with fecal matter as in shrimp *E. hepatopenaei* (Tang et
204 al. 2016). However, extensive detachment of the epithelium and sloughing of tissue
205 ribbons, like in the GSB-infecting myxozoan *Enteromyxum leei* (Sitja-Bobadilla &
206 Palenzuela 2011) are not commonly observed in this infection. More likely, the results
207 point to a late and relatively modest sporulation prolificacy within the fish. Clearance
208 of spores by phagocytes and accumulation in MMC was observed, but it was mostly
209 found in advanced infections with little parasite load and activity. Although *E.*
210 *nucleophila* is rooted in a clade of crustacean-infecting enterocytozoonids (Palenzuela
211 et al. 2014) in which copious sporulation is the norm, the existence of alternate hosts
212 for this species is yet unknown.
213 In summary, the ISH procedure developed in this work appears as a valuable tool for
214 the confirmatory diagnosis of *E. nucleophila* infections in GSB. Although calcofluor
215 staining is an easy method to detect spores, their presence and amount was often

found to be disassociated of the true infection intensity in terms of number of infected host cells. Using the ISH in combination with calcofluor fluorescence staining and conventional histopathological techniques in a stock of clinically infected GSB, we found that the parasite restricts its proliferation and development to the intestine epithelium although isolated spores can reach peripheral blood, and occasionally other digestive and blood-rich organs. Determination of more precise parasite dissemination pathways and pathogeny mechanisms depend on the ability to conduct experimental infections and time-course infection analyses with this parasite.

CONFLICT OF INTEREST STATEMENT

The Authors declare that there is no conflict of interest.

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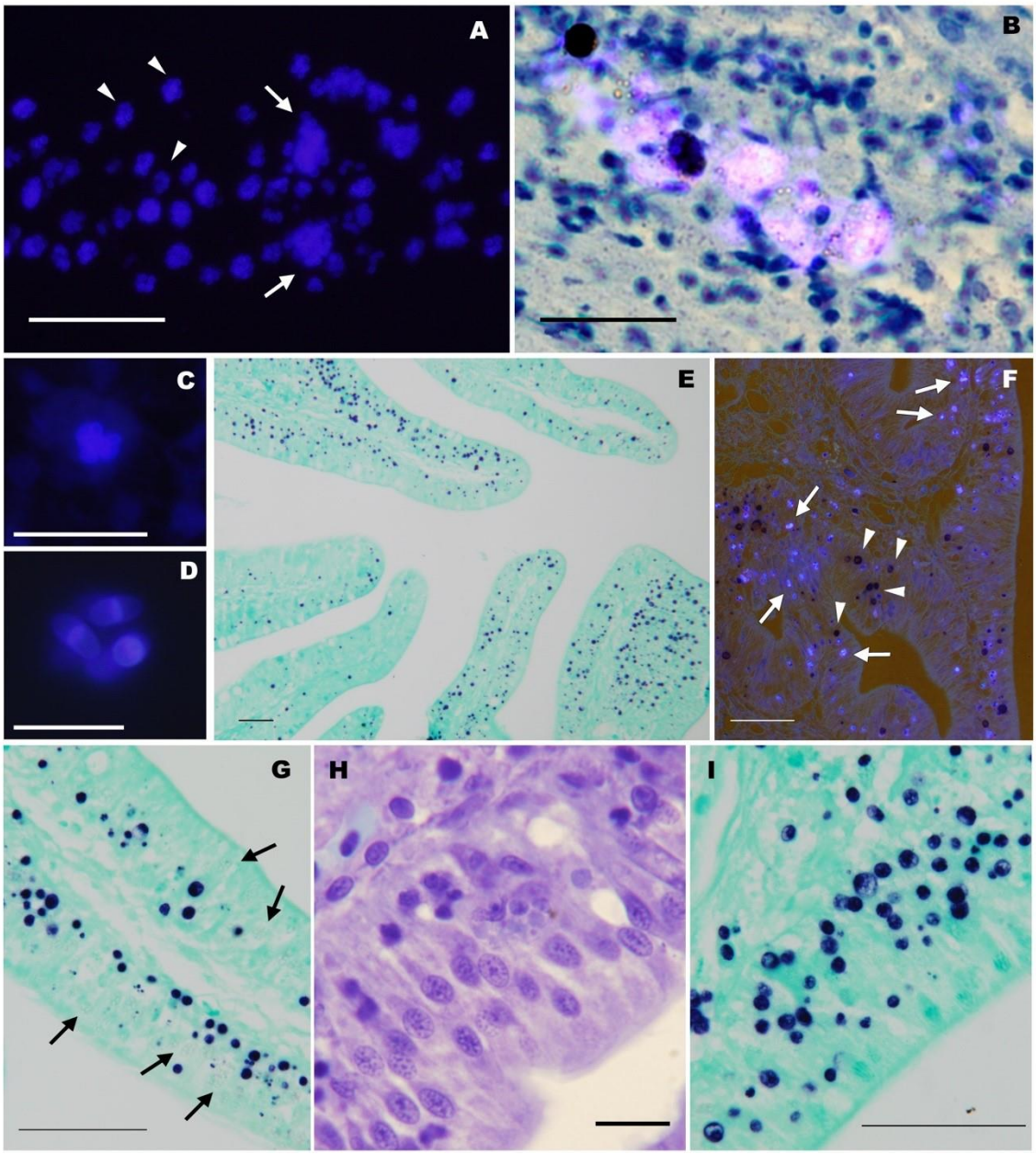
354

355 FIGURE LEGENDS

356 Figure 1: Histology sections of gilthead sea bream tissues infected by *Enterospora*
357 *nucleophila*. A: Infected intestine epithelium showing clusters of intranuclear
358 (arrowheads) and cytoplasmatic (arrows) spores (calcofluor white stain, CW). B: Detail
359 of a melanomacrophage center (MMC) at the intestinal submucosa. Note the bright
360 calcofluor-positive material accumulated within the MMC and in some microsporidian
361 spores at the periphery (double stain with toluidine blue and CW). C & D: *Enterospora*
362 *nucleophila* spores in the gall bladder and the blood, respectively (CW). E: Panoramic
363 view of a heavily infected intestine stained with the ISH procedure, showing
364 conspicuous purple positive cells contrasting with the light green counterstain. F:
365 Panoramic view of an infected intestine. Note the different staining patterns with the
366 ISH (strongly positive cells containing replicating stages stained as deep purple,
367 arrowheads) and the CW (bright blue clusters of mature spores, arrows) (double stain
368 with CW and ISH, photographed under simultaneous visible and UV light). G: Detail of
369 an intestinal epithelium containing –different developmental stages of *E. nucleophila*.
370 Mature spores are mostly unstained (arrows). H: Toluidine blue-stained plastic section
371 showing the typical hypercellularity and altered nuclei often associated with *E.*
372 *nucleophila* infection. I: Detail of ISH-staining pattern in a similar infection to that
373 shown in H. Note the ISH signal with variable intensity in different cells, proportional
374 to the parasite activity and concentrating within the cell nuclei. Scale bars: 20 µm (A &
375 B);
376 10 µm (C); 5 µm (D); 50 µm (Figs. E, G & I); 25 µm (F); 10 µm (H).
377



381

7. *Enterospora nucleophila* and *Cryptosporidium molnari* occurrence in gilthead sea bream mariculture: occurrence and risk factors associated with the detection of the two enteric parasites in the Mediterranean area.

Gilthead sea bream is one of the most bred fish species in the Mediterranean Sea and, together with the sea bass, it represents more than 95% of Mediterranean cultured fish production. Parasitic diseases constitute a key constraint to productivity, economical sustainability and environmental impact of gilthead sea bream husbandry (Timi and Mackenzie, 2015).

Among numerous parasites affecting cultured gilthead sea bream, great importance is attributed to gastro-intestinal unicellular parasites such as myxozoan *Enteromyxum leei*, microsporidian *Enterospora nucleophila* and protozoan *Cryptosporidium molnari*. While *E. leei* impact on gilthead sea bream aquaculture has been widely studied and control strategies as well as effective therapies has been formulated (Sitjà-Bobadilla *et al.*, 2007; Estensoro *et al.*, 2010; Calduch-Giner *et al.*, 2012; Golomazou *et al.*, 2014; Picard-Sánchez *et al.*, 2020a), *C. molnari* and *E. nucleophila* are still little known.

The presence of *E. nucleophila* and *C. molnari* in Spanish farming sites has been ascertained, however there are no available data on their distribution in Mediterranean mariculture, except for some sporadic reports (Caffara *et al*., 2014a; Caffara *et al.*, 2014b; Scaturro, 2015; Gustinelli *et al.*, 2016; Mazzone *et al.*, 2019a; Mazzone *et al.*, 2019b).

The aim of this study was to confirm the presence of *E. nucleophila* and *C. molnari* in gilthead sea bream aquaculture in some of the most important producer countries of the Mediterranean area and to identify potential risk factors that could contribute to the development and detection of both parasites within the rearing system.

The findings of this work may direct future research aimed at studying these parasitic diseases according to an identification of risk factors-based approach and the subsequent phases of risk analysis and management. This study could also assist Public Health policy-makers in developing effective control strategies for parasitic disease in Mediterranean mariculture and thereby in complying with the new Regulation (EU) 2016/429 of the European Parliament on transmissible animal diseases and animal health.

7.1 Materials and Methods

7.1.1 Study population and description of data

The study was carried out from October 2017 to March 2020 at aquaculture facilities located in coastal areas of four Mediterranean countries (Italy, Croatia, Greece and Tunisia). Five-hundred-two fish samples were collected from 17 farms where increased mortality, reduced growth or gastrointestinal clinical signs were previously recorded.

Of these, 123 samples were from 3 land-based farms where early production stages were carried out (hatchery to pre-growing), 2 of which supplied fish eggs, algae, nauplii of *Artemia salina* and rotifers (the latter were fed to larvae) for the analysis.

The remaining 379 samples came from 14 ongrowing farms, where fish were reared until they reached marketable size. Ongrowing farms were represented by inshore sea-cages facilities, land-based and coastal concrete ponds farms. The surveyed facilities, culture system and number of collected samples are shown in Table 1.

Table 1. Farms from the Mediterranean area participating to the study from. Surveyed facilities employed different culture systems. In order to protect farms' anonymity, each site is represented by a distinct alphanumeric code, where letter "C" and the following number represent the Country where the farm was located. Culture systems are identified according to the stage carried out in each farming site: H to PO indicates the steps of hatchery, post-larval, nursery and pre-growing; O indicates ongoing step that raises fish from juveniles to marketable size. The kind of facility is reported following the culture system. *farms which had supplied fish eggs, algae, nauplii of *Artemia salina* and rotifers.

Farm Code	Culture system	No. of Samples	Farm Code	Culture system	No. of Samples
C1F1	O - Ponds	30	**C1F10**	O - Lagoon	3
C1F2	O - Sea Cages	10	**C1F11**	O - Sea Cages	3
C1F3	O - Sea Cages	70	**C1F12**	O - Sea Cages	3
C1F4	O - Sea Cages	6	**C2F1**	H to PO - Tanks	25*
C1F5	O - Ponds	5	**C2F2**	O - Sea Cages	74
C1F6	O - Ponds	11	**C3F1**	O - Sea Cages	8
C1F7	H to PO – Tanks	52*	**C4F1**	H to PO – Tanks	40
C1F8	O - Ponds Seawater	22	**C4F2**	O - Ponds Seawater	10
C1F9	O - Sea Cages	130			

Aquatic animal health professionals from each sample site were asked to record fish weight at sampling time and to provide additional information concerning the tank, pond, or cage where fish had been reared. The collected information pertained the kind of rearing system (net-pen cage, pond or tank), identification code of the batch, rearing stage (hatchery, nursery, weaning, pre-growing and ongrowing), related feeding system and whether they had noticed any clinical signs few days before the sampling at batch level. Table 2 (reported in "Results" section) contains the list of data collected and the number of samples by each variable.

We performed descriptive statistics of the collected data and provided the estimated proportion for *E. nucleophila* and *C. molnari* presence by variable and estimated the related confidence intervals (CIs; at 95% confidence).

Part of the samples collected during this period was used to set up an In Situ Hybridization (ISH) technique aimed at identifying the developmental stages of *E. nucleophila* in intestine histologic sections of infected gilthead sea bream. This work

carried out in collaboration with Dr Palenzuela (CSIC, Spain) was the subject of a scientific publication with is reported at the end of the thesis (Ahmed *et al.*, 2019).

7.1.2 Laboratory diagnostics

7.1.2.1 Sample preparation for molecular analyses

Fish samples collected at different farming sites were sent to the fish pathology laboratory at Department of Veterinary Medical Sciences (DIMEVET) of Bologna University for parasitological examination.

Depending on fish age or size, larvae, fry, juvenile fish and fresh intestinal packages were sent; samples were kept refrigerated during transport.

In order to perform DNA extraction for the subsequent molecular analyses, samples were processed according to the following protocol:

- *Artemia salina* nauplii, rotifers, fish eggs and larvae: the whole sample was placed into a sterile 1,5 ml tube with 0,5 ml up to 1 ml of TE buffer. These samples were processed as pools.
- Fish larvae: larvae's head was excised, and the body was placed in a sterile 1,5 ml tube with 0,5 ml up to 1 ml of TE buffer.
- Fries: the whole gastrointestinal tract was extracted and placed into a single sterile 1,5 ml tube with 1 ml of TE buffer.
- Juveniles and adults: the entire gastrointestinal tract was extracted. A small portion of the stomach was cut and put into a sterile 1,5 ml tube with 0,5 ml up to 1 ml of TE buffer. The intestine was cut lengthwise in order to expose the entire lumen, then the mucosal surface was deeply scraped using a sterile scalpel. The intestinal scraping was put into a sterile 1,5, 2 or 15 ml tube with 1 up to 2,5 ml of TE buffer, depending on the amount of scraped material.

The contents of tubes were manually homogenized and then mixed by vortexing for 5 seconds.

In order to perform DNA extraction, 200 µl of tubes' contents was transferred into a new sterile 1,5 ml tube. DNA extraction was carried out using PureLink Genomic DNA Kit (Life Technologies) and following the manufacturer's protocol.

7.1.2.2 Molecular detection of *Enterospora nucleophila*

In order to detect *E. nucleophila* from DNA specimens extracted from all the collected samples, a Real Time PCR experimental protocol which employed SYBR Green chemistry was assessed.

The assay utilized the primers pair ENU-101F23 5'-TGCGCTAATTTGCTTTATACGAG-3' and ENU-151R22 5'-ATTGCACCTTATTCTACACTCT-3' targeting the 16S rDNA gene of *E. nucleophila*.

The primer pairs were designed from an alignment of available Microsporidia SSU rDNA genotypes, including more than 200 close microsporidians within the family Enterocytozoonidae (Picard-Sánchez *et al.*, 2020b).

A DNA sample previously detected as positive for *E. nucleophila* through sequencing was used as positive control for standard curve drawing. Standard curves were plotted by the amplification of 10-fold dilutions covering 3 orders of magnitude, in triplicate.

Only data from reactions plates with standard curves with an efficiency range between 0.95 and 1.05, and $R^2 > 0.98$ were accepted.

All samples were amplified in duplicates and the mean Ct (threshold cycle) was used for further analysis. Three reactions with a non-template negative control to check for primer-dimer and no specific amplification were also included. Samples with $Ct < 37$ were considered positive. Melting curves of the amplified products and positive control samples were plotted and checked at every qPCR analysis for verifying the presence of one specific peak and the absence of primer dimer and non-specific peaks.

In order to unequivocally assign positive qPCR samples to *E. nucleophila*, a ~1200 bp fragment of 16S rDNA was amplified from qPCR positive specimens. A traditional PCR assay targeting a locus within 16S rDNA was assessed using microsporidian taxon-specific primers 18_f (5'-CACCAGGTTGATTCTGCC-3') and 1492_r (5'-GGTTACCTTGTTACGACTT-3'), according to Weiss and Vossbrinck (1999).

PCR products of were resolved on 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5 × TBE (Molecular Probes - Life Technologies). Positive electrophoresis bands were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel) and sequenced with an ABI 3730 DNA analyser at StarSEQ GmbH (Mainz, Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Thermo Fisher Scientific) and then subjected to BLAST search.

As stated before, sequenced samples matching *E. nucleophila* sequences on GenBank have been used as positive control in further Real Time PCR analyses.

7.1.2.3 Molecular detection of *Cryptosporidium molnari*

In order to detect *Cryptosporidium* spp. from DNA samples, a Nested PCR assay targeting a ~850 bp fragment of the SSU rRNA gene was assessed, according to Caffara *et al.* (2013) and Fayer and Xiao (2007). Primers C1F (5'-TTCTAGAGCTAATACATGCG-3') and C1R (5'-CCCTAATCTTTCGAAACAGGA-3') were used in the primary PCR, where DNA extracted from fish samples was used as template. Secondary PCR employed primers C2F (5'-GGAAGGGTTGTATTTATTAGATAAAG-30) and C2R (5'-AAGGAGTAAGGAACAACCTCCA-3') and primary PCR product as template.

Secondary PCR products were resolved on 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5 × TBE (Molecular Probes - Life Technologies).

In order to identify at species level, the bands of PCR positive samples were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel) and sequenced with an ABI 3730 DNA analyser at StarSEQ GmbH (Mainz, Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Thermo Fisher Scientific) and then subjected to BLAST search.

7.1.3 Univariable statistical analysis

Descriptive statistical analysis and univariate analysis were carried out using in R 4.0.2 (R Core Team 2020). In order to perform statistical analyses, molecular diagnostic results were considered as the outcome variable (or response variable), and the two diseases were studied separately. Positive tested samples were considered as "affected", whereas negative tested samples were considered as "non-affected". Furthermore, anamnestic data collected with the fish samples were elaborated and treated as explanatory variable in further analyses.

As regarding descriptive statistics, proportions of positive samples within different levels of each explanatory variable were calculated and the related confidence intervals (CI) were estimated using Clopper-Pearson confidence interval for a binomial proportion. CI figures were calculated using the *binom.test* function, which is part of the native *stats* package in R 4.0.2 (R Core Team 2020).

As concerns univariable statistical analyses, Fisher's exact test was used for testing the independence between the explanatory and the outcome variable for categorical variables. Additionally, univariate logistic regression was used to understand the relationship between the explanatory and the outcome variable. For continuous variables, a two-sample t-test was used to determine whether there was a significant difference between the means of continuous explanatory variable in affected and non-affected groups. Additionally, univariate logistic regression was used to better understand the relationship between the two variables. In addition to the weight, stage, and type, other independent variables of potential interest were: season, recorded clinical sign (at batch level), whether or not clinical sign was present (at batch level), and whether or not clinical signs were recorded.

7.2 Results

7.2.1 Descriptive test results

The total numbers of samples and the proportion of tested positive samples are presented in Table 2. Totally, 185 individual samples tested positive for *E. nucleophila* and 54 tested positive for *C. molnari*. The estimated proportions of positive samples over the total of collected samples were 0.37 (95% confidence interval (CI): 0.33-0.41) for *E. nucleophila* and 0.11 (95% CI: 0.08-0.14) for *C. molnari*.

Samples from the 17 farming sites were grouped basing on farm geographical location into Western Mediterranean area, which encompasses Italian and Tunisian

facilities, and Eastern Mediterranean area, which encompasses Croatian and Greek facilities. Regarding the distribution, both parasites were detected from both Eastern and Western Mediterranean areas. *E. nucleophila* was detected in 7 (41.2%) farming sites, whereas *C. molnari* were detected in 8 (47%) farming sites; 5 farming sites tested positive for both parasites (29.4%).

The proportion of positive samples for *E. nucleophila* were 0.40 (95% CI: 0.35-0.45) in Western Mediterranean area and 0.30 (95% CI: 0.23-0.38) in Eastern Mediterranean area. The proportions of positive samples for *C. molnari* were 0.12 (95% CI: 0.09-0.16) and 0.08 (95% CI: 0.04-0.14) in Western and Eastern Mediterranean areas, respectively.

Regarding type of rearing and stage of production, the majority of *E. nucleophila* positive samples were from sea-cages, with more than half of samples from sea cages testing positive (0.61, 95% CI: 0.55-0.66) and a similar proportion of positive samples at ongrowing stage (0.48, 95% CI: 0.43-0.53). Very few detections were observed at early rearing stages, with only 1 specimen detected as positive for *E. nucleophila* (0.03, 95% CI: 0.0008-0.16) and 2 (0.06, 95% CI: 0.008-0.21) for *C. molnari* at nursery stage.

On the other hand, *C. molnari* was predominantly detected at pre-growing stage, with an estimate of 0.27 (95% CI: 0.17-0.39), whereas estimate of 0.08 (95% CI: 0.06-0.12) was assessed at ongrowing stage. Proportion of *C. molnari* positive samples was 0.08 (95% CI: 0.05-0.11) at sea-cages. Estimates for ongrowing sites which employed concrete ponds as rearing system were higher for *C. molnari*, with a proportion of positives samples of 0.11 (95% CI: 0.05-0.2) against a proportion of 0.02 (95% CI: 0.003-0.09) assessed for *E. nucleophila*.

Both parasites were detected throughout the years. While the majority of *E. nucleophila* positives specimens were collected in autumn and winter (0.46, 95% CI: 0.38-0.53), the estimate percentages of *C. molnari* positives were greatly higher in warmer seasons (0.17, 95% CI: 0.13-0.22) than in colder seasons (0.03, 95% CI 0.01-0.06). Figure 1 shows the distributions of fish weight (gram) by test results for *E. nucleophila* and *C. molnari* separately. Prior to perform univariate and multivariate analysis, fish weight was grouped into 5 levels: "weight <1.5 g", "1.5 ≤weight <5 g", "5 ≤weight <20 g", "20≤ weight <100g", and "weight ≥100g" (Table 2). Clinical signs were recorded as a presence or absence of the following signs: abdominal swelling, anorexia, weight loss, decreased growth, increased mortality, anemic gills, presence of clinical sings, and whether or not clinical signs are recorded.

Regarding non-fish samples, such as eggs, algae, *A. salina* nauplii and rotifers were always negative for the considered parasites.

Table 2. Laboratory test results and additional information recorded at each sample event are reported. Categories of each variable are represented as they were grouped for further analyses. Clinical signs were recorded considering the batch fish samples belonged to; thus, they are not to be related to the single fish. "Pres" and "Abs" indicate whether the considered clinical sign was recordable at sample time. Percentages of positive tested samples (reported in brackets) were estimated over the total of samples within the considered category.

Variable	Categories		No. of Samples	*E. nucleophila* positive (%)	*C. molnari* positive (%)
Geographic Area	Western Med		345	138 (40%)	41 (12%)
	Eastern Med		157	47 (30%)	13 (8%)
Stage of production	Hatchery + Nursery		32	1 (3%)	2 (6%)
	Weaning		17	0	0
	Pre-growing		74	0	20 (27%)
	Ongrowing		379	184 (49%)	32 (8%)
Type of rearing system	Indoor Concrete Tanks		123	1 (1%)	22 (18%)
	Sea Cages		298	182 (61%)	23 (8%)
	Ponds-Seawater + Lagoon		35	0	9 (26%)
	Ponds		46	2 (4%)	0
Season	Autumn + Winter		222	103 (46%)	7 (3%)
	Spring + Summer		280	82 (30%)	47 (17%)
Year	2017		65	65 (100%)	3 (5%)
	2018		235	79 (28%)	20 (9%)
	2019		163	25 (15%)	31 (19%)
	2020		39	16 (41%)	0
Weight	<1.5 g		73	1 (1%)	2 (3%)
	1.5 g<w<5 g		62	11 (18%)	27 (44%)
	5 g<w<20 g		199	104 (52%)	21 (11%)
	20 g<w< 100 g		66	59 (89%)	3 (5%)
	w>100 g		96	9 (9%)	1 (1%)
Clinical Signs	Abdominal Swelling	Pres	15	2 (13%)	0
		Abs	487	189 (39%)	54 (11%)
	Anorexia	Pres	59	16 (27%)	9 (15%)
		Abs	443	169 (38%)	45 (10%)
	Weight Loss	Pres	105	59 (56%)	13 (12%)
		Abs	397	126 (32%)	41 (10%)
	Decreased Growth	Pres	53	26 (49%)	13 (30%)
		Abs	449	159 (35%)	41 (9%)
	Increased Mortality	Pres	52	47 (90%)	14 (27%)
		Abs	450	138 (31%)	40 (8%)
	Anemic Gills	Pres	84	63 (75%)	5 (6%)
		Abs	418	122 (29%)	49 (11%)
	Clinical Signs not present		215	22 (10%)	22 (10%)

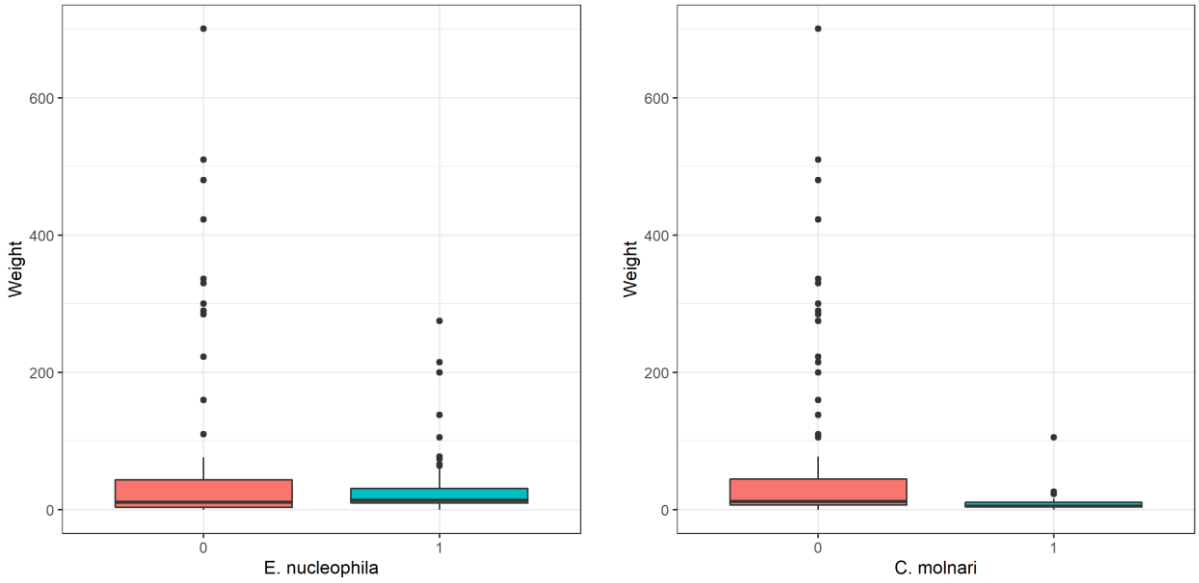


Figure 1. Boxplots representing fish weights and laboratory test results for *E. nucleophila* (left) and *C. molnari* (right). Red boxes represent fish weights distribution within the non-affected group, whereas green boxes represent fish weights distribution within the affected group. Weight on Y axis is in grams.

7.2.2 Univariable analysis

Tables 3 and 4 show the results from the univariable analyses for a significant association between each explanatory variable and *E. nucleophila* detection (Table 3) and *C. molnari* detection (Table 4), respectively.

7.2.2.1 *Enterospora nucleophila*

Univariable analysis showed a statistically significant association between the detection of *E. nucleophila* and weight, geographical area, type of rearing system, stage of production, season, weight loss, increased mortality, anaemic gills, and presence of symptoms at the 5% significance level (Table 3).

As regarding rearing systems, detection of *E. nucleophila* was significantly lower in tanks and ponds compared with cages, and the detection was significantly lower in hatchery, nursery, weaning (here considered as one step comprehensive of the three phases) and pre-growing stages compared with ongrowing stage. Even if there was a statistically significant relationship between *E. nucleophila* and type of rearing system and stage of production, in both cases the estimated odds ratios and confidence intervals were extreme because of the small sample bias and this resulted in low power of the tests. Thus, little can be concluded about the relationships between the probability of detecting *E. nucleophila* positive samples and the different stages of production and different kinds of rearing systems, basing on the data in consideration. As concerns fish samples weight, weight was significant both in continuous and categorical forms. However, issues with

sample bias were again present in the categorical weight with extreme odds ratios and confidence intervals. Season played a significantly role in which autumn/winter has a significantly higher detection of *E. nucleophila* than spring/summertime. Moreover, the presence of some clinical signs such as weight loss, increased mortality, and anemic gills increased the chance of *E. nucleophila* detection by 2.76, 21.25, and 7.28, respectively.

As concern the simultaneous presence of the two parasites, data analyses did not show a statistically significant association between *E. nucleophila* and *C. molnari*.

Table 3. Univariable analyses. Independence tests results evaluating the association between explanatory variables and *E. nucleophila* laboratory tests results as the response variable. "Weight 2" represents the categorical variable obtained by grouping the recorded values of weight into five weight classes.

Variable	Categories	Odds Ratio	95% CI	p-value
Weight		1	0.99-1	0.00022
Geographic Area	East	base		
	West	1.56	1.047-2.35	0.031
Type of rearing system	Cage	base		
	Tank	0.005	0.0003-0.024	2.03e-07
	Ponds	0.016	0.003-0.053	1.30e-08
Stage of production	Ongrowing	base		
	Hatchery to weaning	2.208e-02	1.24e-03-0.10	0.0002
	Pre-growing	9.164e-09	3.13e-129-7066.67	0.98
Season	Autumn/Winter	base		
	Spring/Summer	0.478	0.330-0.691	8.85e-05
Weight	w< 1.5 g	base		
	1.5 g< w < 5 g	12.6	2.34-234	0.017
	5 g< w < 20 g	65.52	14.04-1168.3	3.93e-05
	20 g< w < 100 g	100.459	20.74-1812.02	7.50e-06
	> 100 g	9.947	1.79- 186.3	0.0317
C. molnari detection	No	base		
	Yes	0.841	0.45-1.51	0.57
Abdominal Swelling	No	base		
	Yes	0.256	0.040-0.94	0.075
Anorexia	No	base		
	Yes	0.603	0.321-1.08	0.10
Weight Loss	No	base		
	Yes	2.76	1.78-4.30	0.000006
Decreased Growth	No	base		
	Yes	1.76	0.99-3.12	0.054
Increased Mortality	No	Base		
	Yes	21.25	9.07-62.28	2.15e-10
Anemic Gills	No	base		
	Yes	7.28	4.32-12.71	4.33e-13
No Clinical Sign present	No	base		
	Yes	0.123	0.073- 0.20	<2e-16

Univariable analysis showed a statistically significant association between the detection of *E. nucleophila* and weight, geographical area, type of rearing system, stage of production, season, weight loss, increased mortality, anaemic gills, and presence of symptoms at the 5% significance level

7.2.2.2 *Cryptosporidium molnari*

Statistically significant associations between the detection of *C. molnari* and weight, type of rearing system, stage of production, season, decreased growth, increased mortality, and presence of clinical signs at the 5% significance level were found (Table 4).

Detection of *C. molnari* was higher in tanks and ponds compared with detection in cages, but the difference was only significant between tanks and cages. The data showed a significantly higher detection of *C. molnari* in the pre-growing stage when compared to the ongrowing stage, but no significant difference between the nursery/weaning stage and the ongrowing stage was observed.

Weight was significant both in continuous and categorical forms. However, issues with sample bias were again present, though less than with *E. nucleophila,* in the categorical weight with extreme odds ratios and confidence intervals.

Season played a significant role in which samples collected during spring/summer had a significantly higher detection of *C. molnari* than those collected in autumn/wintertime. The presence of clinical signs of decreased growth and increased mortality increased the chance of *C. molnari* detection by 3.23 and 3.78, respectively.

Table 4. Univariable analyses. Independence tests results evaluating the association between explanatory variables and *C. molnari* laboratory tests results as the response variable. "Weight 2" represents the categorical variable obtained by grouping the recorded values of weight into five weight classes.

Variable	Categories	Odds Ratio	95% CI	p-value
Weight		0.966	0.939-0.986	0.00627
Geographic Area	East	base		
	West	1.494	0.796- 2.98	0.229
Type of rearing system	Cage	base		
	Hatch/Pre-grow	2.604	1.385-4.891	0.00279
	Ponds	1.495	0.632-3.271	0.33275
Stage of production	Ongrowing	base		
	Nurse/weaning	0.461	0.073-1.59	0.299
	Pre-grow	4.016	2.121-7.491	1.43e-05
Season	Autumn/Winter	base		
	Spring/Summer	6.2	2.92- 15.28	1.17e-05
Weight	w< 1.5 g	base		
	1.5 g< w < 5 g	16.6	4.58-106.76	0.000235
	5 g< w < 20 g	4.32	1.24-27.39	0.05
	20 g< w < 100 g	1	0.16-7.76	1
	> 100 g	0.48	0.022-5.099	0.55
E. nucleophila detection	No	base		
	Yes	0.841	0.454-1.51	0.571
Abdominal Swelling	No	base		
	Yes	5.12e-07	NA-1.7e +11	0.981
Anorexia	No	base		
	Yes	1.59	0.69-3.32	0.239
Weight Loss	No	base		
	Yes	1.23	0.61-2.33	0.546
Decreased Growth	No	base		
	Yes	3.23	1.56-6.41	0.00107
Increased Mortality	No	Base		
	Yes	3.78	1.84-7.44	0.00017
Anemic Gills	No	base		
	Yes	0.477	0.162-1.13	0.127
No Clinical Sign present	No	base		
	Yes	0.392	0.188-0.754	0.0077

7.3 Discussion

The present work confirms the presence of the two parasites in gilthead sea bream (*Sparus aurata*) aquaculture in the Mediterranean basin. In particular, this is the first report of both *E. nucleophila* and *C. molnari* from Eastern Mediterranean culture systems and the first work investigating the simultaneous presence of the two enteric parasites.

Regarding *E. nucleophila*, no other epidemiological studies that analyze its presence and distribution within the production cycle of gilthead sea bream have been published to date. Given the recent first report (Palenzuela *et al.*, 2014) and the great

challenges in detecting microsporidians as a general statement, the available works mainly focus on the evaluation of innovative and effective detection methods (Ahmed *et al.*, 2019; Picard-Sánchez *et al.*, 2020b) and on the study of *E. nucleophila* life cycle within the type host gilthead sea bream (Palenzuela *et al.*, 2014).

The only data concerning the presence of *E. nucleophila* within different rearing systems showed a diffuse presence among different types of ongrowing facilities. Positive specimens were detected in all the sampled farms, consisting in either sea-cages, concrete ponds, and earth ponds farms (Palenzuela *et al.*, 2014; Ahmed *et al.*, 2019; Picard-Sánchez *et al.*, 2020b). Comparing studies about *E. nucleophila* with other microsporidians affecting gilthead sea bream is rather controversial, since most of the few species described both in wild and cultured fish did not target intestinal epithelium neither had intranuclear development (Faye *et al.*, 1990; Abela *et al.*, 1996; Athanassopoulou, 1998; Fioravanti *et al.*, 2006; Morsy *et al.*, 2013). Moreover, lesions associated with these microsporidians presence in gilthead sea bream were not sparse within the host tissues, but they are characterized by xenoma's presence in muscles or body cavities (Morsy *et al.*, 2013).

The presence of *E. nucleophila* from hatchery to market size has been recently studied in a three-year's survey carried out in two different Mediterranean countries whose results have not been published yet. However, confidential preliminary data demonstrated positivity percentages at nursery stage sensibly higher than what we observed (only one positive sample at this stage). Moreover, they found up to 100% of positivity among sampled fish at pre-growing stage, which is not consistent with the observation of this study, since no positive sample was detected at pre-growing stage.

Palenzuela *et al.* (2014) reported a seasonality in the appearance of clinical signs within affected sea bream, since the emaciative syndrome related to *E. nucleophila* infection was mostly observed during the first winter fish spent in sea cages. This aspect of the disease seemed to be confirmed by the statistical correlation observed between different seasons and *E. nucleophila* detection, since samples collected during autumn and winter had a significantly higher detection of *E. nucleophila* than those collected in warmer seasons.

As for *C. molnari*, the only other epidemiological survey investigating the protozoan's presence throughout the whole production cycle of gilthead sea bream is the work by Sitjà-Bobadilla *et al.* (2005).

Regarding the different production stages, the proportions of positive samples observed in our work were consistent with what reported by Sitjà-Bobadilla *et al.* (2005). In the present survey, only two samples were detected as positive at hatchery and nursery stages in accordance with Sitjà-Bobadilla *et al.* (2005), who reported that fish were rarely infected before weaning since positive samples were detected in only two of the examined batches.

The highest percentages of positivity were observed at pre-growing stage (27% of positive samples), which is consistent with the range of positivity of 10-25% reported by Sitjà-Bobadilla *et al.* (2005).

At the same time, the authors reported high positivity rates for the first samplings at ongrowing facilities, just after cages, tanks or concrete ponds had been seeded. These data could not be compared with those presented in this survey, due to the small sample size and the absence of batches follow-up through further samplings. The observed differences between positivity rates at different stages of production were confirmed by univariable statistics analysis, since a statistically significant higher detection of *C. molnari* at pre-growing than at other stages was observed. Both Sitjà-Bobadilla *et al.* (2005) and Alvarez-Pellitero *et al.* (2009), the latter in an epidemiological survey on *C. scophthalmi* presence in turbot (*Scophthalmus maximus*) production cycle, hypothesized that this difference may be related to water quality, as treatments of the water supply usually change substantially from the hatchery, post-larval and nursery steps, where water is always filtered (fine and coarse filtration, depending on the step) to pre-growing steps, where water supply usually undergoes only coarse filtration or it is not filtered at all. Moreover, aquaculture procedures using recirculation systems and cannibalism among fingerling and juvenile fish may contribute to concentrate oocysts and facilitate parasite transmission (Sitjà-Bobadilla *et al.*, 2005).

As concerns the type of facility, the present survey showed higher detection at tanks and ponds facilities than at sea-cages farms. While any statistically significant difference was found among different ongrowing types of facilities by the work of Sitjà-Bobadilla *et al.* (2005), univariable analysis carried out in this work showed statistically significant differences between tanks and cages rearing systems. The discrepancies between our results and the data reported by Sitjà-Bobadilla *et al.* (2005) et al me be related to different water filtration and sterilisation systems and different tanks routinely cleaning schedules in the analysed farming sites, which may have led to a major persistence of infectious oocysts in the rearing system.

As regards the distribution of positive samples among different fish weights classes, Sitjà-Bobadilla *et al.* (2005) reported the highest proportion of positive fish in the 30 g to 100 g fish class, with infection levels subsequently decreasing in larger animals and disappearing in fish weighing more than 300 g. This data is not consisted with what we observed in this study, since the higher percentages of positive samples were observed among fish weighting between 5 g and 20 g.

Sitjà-Bobadilla *et al.* (2005) also observed a statistically significant decreasing trend of positivity rates with increasing fish weight at ongrowing stage of production, which was not observed in the present work, probably due to the small size of the sample.

Concerning *C. scophthalmi* detection and fish weight, Alvarez-Pellitero *et al.* (2009) observed over a period of several years, high prevalence of infection in small fish, with a sharp decrease in larger animals. The latter data are more consistent with the findings of this study, since only four fish with weight > 20 g were detected as positive.

Sitjà-Bobadilla *et al.* (2005) reported a seasonal distribution of *C. molnari* positive specimens, with maximum prevalence estimated in samples collected in spring, followed by summer. This was confirmed by the strong correlation observed between season and *C. molnari* detection, since samples collected during spring and summer had a significantly higher detection of *C. molnari* than those collected in autumn and wintertime. Temperature is probably one of the major factors involved in seasonal fluctuations of *C. molnari* infection since it is scarcely controllable in outdoor rearing systems.

Data presented in this survey showed that pre-growing stage, tank rearing system and warm seasons significantly increased the probability of detecting positive samples. They can be considered as risk factors for the detection of *C. molnari* and worthy of more detailed studies. Despite a strong correlation between the presence of some clinical signs such as increased mortality and decreased growth rates and the detection of *C. molnari*, the sampling schemes and collection of data, which were recorded at batch level, did not allow to further speculate on the correlation between clinical signs and probability of detection. The same can be applied to weight.

As concerns the simultaneous presence of *E. nucleophila* and *C. molnari* in the surveyed facilities, almost one third of the farms tested positive for both parasites. Despite significance tests did not show any statistical correlation between the detection of *E. nucleophila* and the detection of *C. molnari*, the present work's finding highlights the need for further investigations on the interaction between the two parasites themselves and the host.

Despite gilthead sea bream and European sea bass are the most farmed marine fish species in the Mediterranean area, knowledge on many emerging parasites in this sector and their impact on productions are lacking. Contrarily to metazoan parasites, which are very often visible by naked eye and therefore easily diagnosed, protozoans and microsporidia are limited in size and more difficult to detect (Buchmann, 2015). When unicellular parasites' infections can be observed macroscopically, this is usually due to pathological tissue changes induced by parasites. Despite the limited size of protozoans and microsporidia, their pathogenic effects on fish may be devastating and can negatively impact on fish production (Buchmann, 2015).

Although the presence of *Cryptosporidium* spp. and *E. nucleophila* in gilthead sea bream farms has been reported sporadically for twenty years, data on their spread in Mediterranean mariculture are still scant and superficial. This may be related to the challenging detection of unicellular parasites combined with the low incidence of the clinical signs among the infected groups of fish, which may have been led to overlook or even neglect *E. nucleophila* and *C. molnari* presence in gilthead sea bream productions (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla *et al.*, 2005; Palenzuela *et al.*, 2014; Picard-Sánchez *et al.*, 2020b).

The present survey contributed to shed light on *E. nucleophila* and *C. molnari* diffusion within Mediterranean gilthead sea bream mariculture. Moreover, the attempt of a risk factors individuation-based method for studying these parasitic diseases in

Mediterranean aquaculture provided few yet interesting findings on where further studies may be directed to. Additional studies will help in investigating potential risk factors for the detection of *E. nucleophila* and *C. molnari* and to evaluate critical control points within gilthead sea bream production cycle, in order to manage the threats arousing from these emerging enteric parasites. Their prevention, control and eradication from Mediterranean mariculture are of paramount importance for the efficient production of cultured gilthead sea bream.

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New advances in detection and molecular characterization of fish-borne zoonotic parasites from European freshwater fish.

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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF LARVAL AND ADULT STAGES OF EUSTRONGYLIDES EXCISUS (NEMATODA: DIOCTOPHYMATOIDEA) WITH HISTOPATHOLOGICAL OBSERVATIONS

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Abstract:	The genus Eustrongylides includes nematodes known as etiological agent of the “Big Red Worm Disease”. The aim of this work was to identify Eustrongylides spp. larvae from fish and adults from great cormorant (Phalacrocorax carbo) sampled at Lake Trasimeno, Italy, by morphological and molecular analysis. Histopathological description of the lesions in birds was also provided. We describe for the first time in Italy adults of Eustrongylides excisus and we also link larval stages (L3 and L4) to adults. The use of molecular tools combined with the traditional taxonomy will help the identification of the species, including species inquirendae. Moreover, the molecular analysis can also help to investigate the role of intermediate and paratenic hosts, to deepen the knowledge about geographical distribution of the different Eustrongylides species and to define the zoonotic potential of E. excisus, which has not yet been identified as causal agent of human cases.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF LARVAL AND ADULT STAGES OF *EUSTRONGYLIDES EXCISUS* (NEMATODA: DIOCTOPHYMATOIDEA) WITH HISTOPATHOLOGICAL OBSERVATIONS

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ABSTRACT

The genus *Eustrongylides* includes nematodes known as etiological agent of the “Big Red Worm Disease”. The aim of this work was to identify *Eustrongylides* spp. larvae from fish and adults from great cormorant (*Phalacrocorax carbo*) sampled at Lake Trasimeno, Italy, by morphological and molecular analysis. Histopathological description of the lesions in birds was also provided. We described adults of *Eustrongylides excisus* for the first time in Italy and we also linked larval stages (L3 and L4) to adults. The use of molecular tools combined

23 with the traditional taxonomy will help the identification of the species, including species
24 inquirendae. Moreover, molecular analysis can also help to investigate the role of
25 intermediate and paratenic hosts, in order to deepen the knowledge about geographical

distribution of the different *Eustrongylides* species and to define the zoonotic potential of *E. excisus*, which has not yet been identified as causal agent of human cases.

KEY WORDS

Eustrongylides excisus; Morphology; Histology; Molecular Identification; Great Cormorant; *Phalacrocorax carbo*

The genus *Eustrongylides* Jägerskiöld, 1909 includes nematodes (Nematoda, Dioctophymatidae) affecting several fish species and piscivorous birds of freshwater ecosystems, causing the "Big Red Worm Disease". The genus *Eustrongylides* includes several species, among which *Eustrongylides tubifex* Nitzsch in Rudolphi, 1819, *Eustrongylides ignotus* Jägerskiöld, 1909 and *Eustrongylides excisus* Jägerskiöld, 1909, are responsible for outbreaks of mortality in nestlings of several orders of birds worldwide (Measures, 1988a; Cole, 1999). Nematodes belonging to the genus *Eustrongylides* are characterized by an indirect life cycle: the first intermediate host is represented by freshwater oligochaetes, whereas numerous benthic fish can serve as the second one, in which infective larvae (L4) are encapsulated into muscles and visceral serosae (Paperna, 1974; Cooper et al., 1978). Predatory fish, amphibians and reptiles can be part of the parasite's cycle as paratenic hosts, accumulating larvae in muscles and/or visceral serosae (Goncharov et al., 2018).

Several species of piscivorous birds act as definitive hosts, among which the great cormorant (*Phalacrocorax carbo* Linnaeus, 1758) has often been reported to host adult worms (El-Dakhly et al., 2012; Stocka et al., 2017). In birds, the parasite is more commonly found in the wall of proventriculus but also in ventriculus and intestine. In these organs the worms' penetration forms large and tortuous tunnels and huge granulomatous response around the parasites themselves (Measures, 1988a; Cole, 1999; Xiong et al., 2009).

In the last few decades there has been a growing attention on *Eustrongylides* spp. nematodes' as zoonotic agents (Gunby, 1982; Wittner et al., 1989; Narr et al., 1996; Eiras et al., 2018). Man becomes infected by the consumption of raw or undercooked fish infected by the larval stages of the parasite, developing gastrointestinal syndromes ranging from mild gastritis or enteritis to stomach and intestinal perforation (Eberhard et al., 1989), and in some cases cutaneous lesions (Eberhard and Ruiz-Tiben, 2014).

Jägerskiöld erected the genus *Eustrongylides* in 1909 providing the first revision of the genus. Karmanova (1968) further revised the taxonomy of *Eustrongylides*, recognizing 14 species 3 of which described only at larval stage. Later on, Measures (Measures, 1988b) established as valid species only *Eustrongylides tubifex*, *Eustrongylides ignotus* and *Eustrongylides excisus* and considered the remaining species as *species inquirendae*.

Eustrongylides species have been described all around the world with different distribution depending on the species. Recently, larval stages of *Eustrongylides* spp. have been reported for the first time in European perch (*Perca fluviatilis* Linnaeus, 1758) from Lake Trasimeno, Italy (Dezfuli et al., 2015). Later Agnetti et al. (2016) and Branciari et al. (2016) reported the presence of *Eustrongylides* spp. in other fish species. Agnetti et al. (2017) and Mazzone et al. (2018) described the infection in cormorant (*Phalacrocorax carbo*) from the same area. The presence of *Eustrongylides* spp. in fish is a matter of concern as an intense fishing activity insists in this area and if fish products are consumed raw or undercooked could represent a threat for human health, besides the obvious impact on the marketability of the fish products.

The aim of this work was to identify *Eustrongylides* spp. larvae from European perch (*Perca fluviatilis*) and Sand smelt (*Atherina boyeri* Risso, 1810) and adults collected from great cormorant (*Phalacrocorax carbo*) sampled at Lake Trasimeno, by morphological and molecular analysis. A complete description of *E. excisus*, the species identified in this study,

is given based on a high number of specimens and supported by microscopical pictures for the first time. Moreover, histopathological description of the lesions in birds was also provided.

MATERIALS AND METHODS

Sixty nematodes, of which 9 larvae from the muscle of 5 European perch (*Perca fluviatilis*) and 2 Sand smelt (*Atherina boyeri*) and 51 adults from the proventriculus of 2 Great cormorants (*Phalacrocorax carbo*) were collected at Lake Trasimeno, Italy (43°05'22.5"N, 12°08'11.3"E). All the specimens were preserved in 70% ethanol. Before clarification with Amman's lactophenol a little portion of the central part of the body was cut for molecular analysis. Morphometries of the hologenophores and paragenophore (sensu Pleijel et al., 2008) were taken, following the key reported by Measures (1988b) with the imaging software NIS-Elements (Nikon, Florence, Italy). Adults were extracted from the granuloma-like structures of the proventriculus, but as they were deeply embedded in the tissues, it was not possible to collect the whole body. For this reason, the total length is not provided. In particular, in 21 specimens both cephalic and caudal portions were collected and studied, in 10 only the cephalic portion was extracted and measured, while the other 20 specimens were not suitable for morphological study, so they were processed only for molecular analysis.

The DNA was extracted with PureLink Genomic DNA Kit (Life Technologies, Carlsbad, California) following the manufacturer's protocol. Amplification of *ITS rDNA* (*ITS1, 5.8S* and *ITS2*) employed protocols and primers (81_f GTAACAAGGTTTCCGTAGGTGAA and ITS2.S_r CCTGGTTAGTTTCTTTTCCTCCGC) reported by Gustinelli et al. (2010). Amplified products were resolved on 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5× TBE (Molecular Probes - Life Technologies, Carlsbad, California). For sequencing, bands were excised and purified by NucleoSpin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany) and sequenced with an ABI 3730

DNA analyser at StarSEQ GmbH (Mainz, Germany). Contigs were assembled with Vector NTI AdvanceTM 11 software (Thermo Fisher Scientific, Carlsbad, California) and sequences are published in GenBank under the following accession numbers MK545492-MK545541. Pairwise distances and models of nucleotide evolution (BIC) were calculated using MEGA 6.06 (Tamura et al., 2013). The K2P model was used for Maximum Likelihood analysis. The newly generated *ITS rDNA* sequences were aligned with *Eustrongylides* spp. available in GenBank. *Pellioditis marina* (AM398823) was used as outgroup.

A portion of the proventriculus infected by *Eustrongylides* was preserved in 10% buffered formalin for histopathological observation. The sections were stained with Hematoxylin-Eosin (HE).

RESULTS

The morphological features of the adults allowed us to identify them as belonging to the species *Eustrongylides excisus*, while the features of larval stages did not lead to species identification. Measurements of adults and larvae are provided in Tables I and II, respectively.

Large nematodes. Broad, tapering at extremities; broadening begins posteriorly to the nerve ring. Cuticle thin transversely striated, most of the specimens showed 3 or 4 layers of cuticle. Cuticle pale and opalescent. Cephalic extremity (Fig. 1A) with 12 labial papillae arranged in two concentric circles, each composed by 6 papillae. Inner circle with 2 lateral, 2 subventral and 2 subdorsal papillae with narrow bases and spine-like tip, which is easily distinguishable from rest of the papilla structure. Outer papillae short, consisting of 2 lateral, 2 subventral and 2 subdorsal papillae, with wide bases (almost twice larger than height) and nipple-like tip. Somatic papillae were arranged in 1 row in each lateral field from anterior to posterior extremity. Somatic papillae near extremities closer together than those near midbody. Buccal cavity narrow and lined with cuticle. Nerve ring close to the end of buccal cavity and not always clearly defined. Rectum short, lined with cuticle.

Male: Posterior extremity (Fig. 1B) expanded into muscular bursa; mean length and mean width at posterior extremity: 477.8 × 949.1 µm. Inner cuticular surface of caudal bursa presented bosses. Bosses near to hem close to each other than those in middle part of the caudal bursal surface. Outer perimeter of bursa with wide cuticular hem and indented. Ventral edge of bursa with deep cleft. Spicule needle-like (Fig, 1D) with simple distal point; spicule average length: 4.75 mm. Muscular sheath of spicule thick, extending well beyond proximal extremity of spicule. In three specimens muscular sheath of spicule was covered by little and tiny spines (Fig, 1C), densely arranged on whole surface.

Female: Posterior extremity blunt (Fig 1E), with terminal anus and vulva. Mean width at posterior extremity 667.1 µm. Vulva ventral to anus, anus smaller than vulva. In 2 specimens the anus presented 3 papillae of variable dimension in 3 samples; each papilla differed in shape and dimensions from another. Vulva thick and muscular. Uterus with indented surface. In 2 specimens the uterus formed 2 close rounded curvatures. Females extracted from granuloma-like structures located in the thickness of the proventriculus presented pink to crimson cuticle; in this specimens uterus was widely enlarged by the massive presence of eggs.

Eggs (n = 50) (Fig. 1F): Oval, flattened at each pole. Shell thick with irregular outer surface. Average dimensions: 67.96 × 39.70 µm.

Morphological description of larval stages (n = 9) (Fig. 2): According to the morphology of labial papillae and to the development of genital primordium (Karmanova, 1968; Measures, 1988c; Xiong et al., 2009) it was possible to distinguish 1 male third stage larva (L3) and 8 (4 male and 4 female) fourth stage larvae (L4). Large nematodes; cephalic extremity tapering (Fig. 2A), body broadening posteriorly to the nerve ring. Pink to red thin cuticle. Cuticle transversely striated; most of them presented 2 or 3 layers of cuticle kept from the previous moult. Cephalic extremity with 12 labial papillae arranged in 2 concentric circles

surrounding oral cavity, each composed by 6 papillae. Both circles' papillae arranged in pairs, 2 lateral, 2 subventral and 2 subdorsal; inner circle papillae and outer circle papillae had approximately the same dimensions. Inner circle papillae presented spine-like apices and narrow bases, whereas outer circle papillae had nipple-like apices and wide bases. Nerve ring could be observed behind the end of buccal cavity, even if it was not always clearly definable. Long esophagus forming several curvatures starting from the end of oral cavity to the beginning of intestine; terminal anus. As previously reported by Karmanova (1968) and Measures (1988c, 1988d), the L3 showed a developing genital primordium, whereas fourth stage larvae (Fig. 2B, C) displayed more developed genital primordia. All the larvae presented 2 rows of somatic papillae arranged in 1 row in each lateral field from anterior to posterior extremity. Somatic papillae near extremities more developed and closer together than that near midbody; some samples did not show somatic papillae at midbody.

PCR successfully amplified the 9 larvae and 41 adults. The *ITS rDNA* sequences of the larvae were 757-822 bp long while the ones from adults 790-826 bp. Sequences were identical to each other (p-distance = 0%) and between larvae and adults. BLAST search gave 99.9% - 96% similarity with *ITS rDNA* of *Eustrongylides* spp. of Xiong et al. (2013) from China and 99.9% with *Eustrongylides* spp. (KU963206, Tabaripour and Youssefi, unpubl and MK007967, Pekmezci and Bolukbas, unpubl) from Iran and Turkey respectively. The Maximum-Likelihood tree (Fig. 3) showed our sequences clustering with clade 3 (p-distance 0%) and with clade 2 (p-distance 0.1-0.2%) of Xiong et al. (2013) and with the sequences from Iran and Turkey. While clade 1 of Xiong et al. (2013) forms a clear separated cluster (pdistance 2.1-2.2%).

Histological sections showed both longitudinal and transverse sections of the parasite (Fig. 4A). Some of the parasites were found on the surface of the glandular mucosa of the proventriculus or deeply embedded into the muscular layer, while others penetrated through

the serosal membrane. Furthermore, some other worms presented either anterior or posterior extremities, protruding through narrow apertures toward the lumen, and then retracting back into the wall.

Tissues surrounding parasite sections showed concentric and different layers of inflammatory cells and pathological alteration (Fig. 4B): immediately around the parasite's body there were 1 or 2 layers of multinucleate giant cells. According to nuclei arrangement (Fig. 4C), 2 types of giant cells could be distinguished: in the first type, nuclei were arranged in a horseshoe-shape at the periphery of the cell itself, while in the second type they were placed over the whole cell surface. Externally to the multinucleate cellular layer there were necrotic areas in which the underlying architecture and tissue specialization was sometimes completely altered; cells in the necrotic areas rarely presented karyorrhexis. Necrotic tissues were surrounded by phlogistic processes (Fig. 4D), characterized by the abundant presence of macrophages, plasma cells and sparse mature lymphocytes mixed with fibroblasts and loose connective tissue. In some areas, the inflammatory process was characterized by the presence of rare eosinophilic granulocytes. Secreting epithelium of the glandular acini presented cellular degeneration phenomena when invaded by parasites (Fig. 4E); moreover, flaked cells mixed with amorphous material occupied glandular lumen. Inflammatory cells occupied the space between cells composing the acini and the lumen. Some areas showed hyperemic vessels: hyperemia was more pronounced where inflammation was characterized by hyperplasia, especially related to an increase of eosinophil granulocytes. Phlogistic areas where hyperemia was less pronounced or absent were also characterized by the presence of mature fibroblasts and few inflammatory cells. Bacteria surrounded by lymphocytes, eosinophilic granulocytes and macrophages were present nearby the mucosal surface were worms had penetrated. Transverse sections of the parasites exhibited several anatomical structures: digestive tract was distinguishable by the presence of a layer of simple columnar

cells; it sometimes showed erythrocytes into the lumen. Female adults displayed gonads and uterus sections, where embryonated eggs were frequently present (Fig. 4F). Wide areas characterized by inflammatory infiltrate were also present in the thickness of proventricular mucosa in every histological preparation. Parasites themselves showed phenomena of tissue degeneration and necrosis, when surrounded by inflammatory reactions with abundance of mature fibrous tissue.

DISCUSSION

In this study, we describe adults of *Eustrongylides excisus* by morphological and molecular analyses for the first time in Italy, also linking the larval stages (L3 and L4) to the adult.

The morphological features suitable to discriminate among *Eustrongylides* species are the caudal end of male and size and shape of the labial papillae around mouth opening. The features observed in our specimens were consistent to *E. excisus* described by Karmanova (1968) and Measures (1988b) despite minor discrepancies. These authors described 4 small and flat lateral-field papillae between inner and outer circles of labial papillae; this pattern has never been observed in our specimens. The inner and outer circle papillae presented similar dimensions, as reported for *E. excisus* and *E. ignotus* by Karmanova (1968) and Measures (1988b), while, *E. tubifex* displays small inner circle papillae and much larger outer circle papillae. Three of the males under study showed small and thin spines densely arranged on the whole surface of the spicule muscular sheath; this feature, to the best of our knowledge, has never been described before. Finally, the caudal bursa of the male displayed a peculiar deep cleft on ventral side, which is absent in both *E. ignotus* and *E. tubifex* but also in all the species inquirendae.

Concerning the larvae collected from fish hosts, as reported by Moravec (1994) and Moravec and Nagasawa (2018), they did not display useful taxonomical features to reach

225 species identification. According to the morphology of labial papillae, our larval stages could
226 be identified as either *E. ignotus* (Karmanova, 1968; Melo et al., 2016) or *E. excisus*
227 (Karmanova, 1968; Moravec, 1994; Moravec and Nagasawa, 2018). Furthermore, male larvae
228 of *E. excisus* do not present the deep cleft characterizing the adult's caudal end, which is
229 distinctive for this species. For these reasons, the link between adult and larvae could be
230 achieved only by comparing the DNA sequences of both developmental stages as reported in
231 this study.

232 Among the studies describing the histological lesions associated with the presence of
233 *Eustrongylides* spp. in birds (Locke, 1961; Locke et al.,1964; Measures, 1988a; Roffe, 1988;
234 Spalding and Forrester, 1993; Ziegler et al., 2000; Pinto et al., 2004; El-Dakhly et al., 2012)
235 none of them was referred to *E. excisus*. The lesions due to *E. excisus* in avian definitive host,
236 described in this study were similar to those caused by *E. tubifex* in great cormorant
237 (*Phalacrocorax carbo*) (El-Dakhly et al., 2012) and by *E. ignotus* in ciconiiform birds
238 (Spalding and Forrester, 1993; Pinto et al., 2004).

239 Although *Eustrongylides* spp. appear to contribute rather than cause death in adult
240 birds (Spalding and Forrester, 1993), the histopathological lesions may explain how this
241 parasite can cause large outbreaks of mortality in nestling wading birds, as previously
242 observed by Wiese et al. (1977).

243 The molecular analysis combined with the morphological description supported the
244 species identification of both adults and larvae as *E. excisus*, making the sequences of this
245 species available. All our sequences grouped in the clade 3 of Xiong et al. (2013). These
246 authors sequenced larvae from different fish hosts, stating that they were phylogenetically
247 grouped in 3 different clades belonging to 2 different species. Nevertheless, no morphological
248 support was provided by these authors. Xiong et al. (2013) speculated that the specimens
249 grouped in clade 1 should be considered as a cryptic species, while the ones comprised in

clades 2 and 3 belong to the same species. The molecular results obtained in this study support this hypothesis, in fact the p-distance values observed, 0-0.2%, allowed us to state that the parasites found in China (clades 2 and 3) are likely to belong to the species *E. excisus*. This hypothesis if further corroborated by Xiong et al. (2013) who reported an intraspecific ITS variation between clade 2 and 3 (considered belonging to the same species) ranging from 1.8 to 2.8%. It is also important to note that the sequences from Iran and Turkey, can be classified as *E. excisus*, based on molecular comparison (p-distance 0.1%). The use of molecular tools combining the traditional taxonomy will be essential in further identifications of *Eustrongylides* species, including species inquirendae. Moreover, molecular analysis can also help to investigate the role of intermediate and paratenic hosts and increase the knowledge about geographical distribution of the various species of these nematodes. The combined use of the above mentioned methods would also be useful in defining the zoonotic potential of *E. excisus*, which has not yet been identified as causal agent of human cases.

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Figure 1. *Eustrongylides excisus*. Adults. (**A**) Cephalic extremity: inner and outer circle labial papillae, lateral-field somatic papillae. (**B**) Male caudal end: bosses and indented hem, deep cleft on the ventral edge of bursa. (**C**) Male caudal end: spines on the muscular sheath of spicule. (**D**) Male caudal end with completely everted spicule. Scale Bar= 1000 µm. (**E**) Female caudal end: vulva and anus, lateral-field somatic papillae. Abbreviations: anus, a; vulva, v. (**F**) Egg.

Figure 2. *Eustrongylides excisus*. Fourth larval stage. (**A**) Cephalic extremity: inner and outer circle labial papillae, lateral-field somatic papillae; oral cavity, nerve ring and oesophagus are also visible. (**B**) Female caudal end: vulva and anus; cuticle. (**C**) Male caudal end: primordia of bursa and muscular sheath of spicule; lateral-field somatic papillae; multiple layers of cuticle. Scale Bars= 100 µm. Abbreviations: anus, a; cuticle, c.; vulva, v.

Figure 3. Evolutionary history inferred using the Maximum Likelihood method based on the Kimura 2-parameter model from ITS rDNA of *Eustrongylides excisus* generated in this study. Only 5 representative sequences from the different developmental stages were used (in bold) as all the 50 sequences were identical. Node are labelled with bootstrap values of 1,000 replicates.). Abbreviations: clade 3, *; clade 2, @; clade 1, § of Xiong et al. (2013).

Figure 4. *Eustrongylides excisus* in Great cormorant proventriculus. Sections stained with Hematoxylin-Eosin. (**A**) Cross-sections of an encapsulated adult female. (**B**) Cross-section of an adult surrounded by multiple layers of inflammatory cells (i), fibrotic tissue (f) and necrotic areas (n). (**C**) Layer of multinucleated cells (*) surrounding the parasite (p). (**D**) Vast necrotic areas (n) and inflammatory infiltration (i) close to the parasites cuticule. (**E**) Degenerated glandular parenchyma: inflammatory cells (i) between acini; flaked cells and amorphous materials in the lumen (*); hyperemic vessel (h). (**F**) Cross-sections of an adult female: digestive tract, uterus filled-with eggs, ovary. Abbreviations: digestive tract, dt; ovary, o; uterus, u. Color version available online.

Figure 1

Click here to access/download;Figure;Fig 1.tif

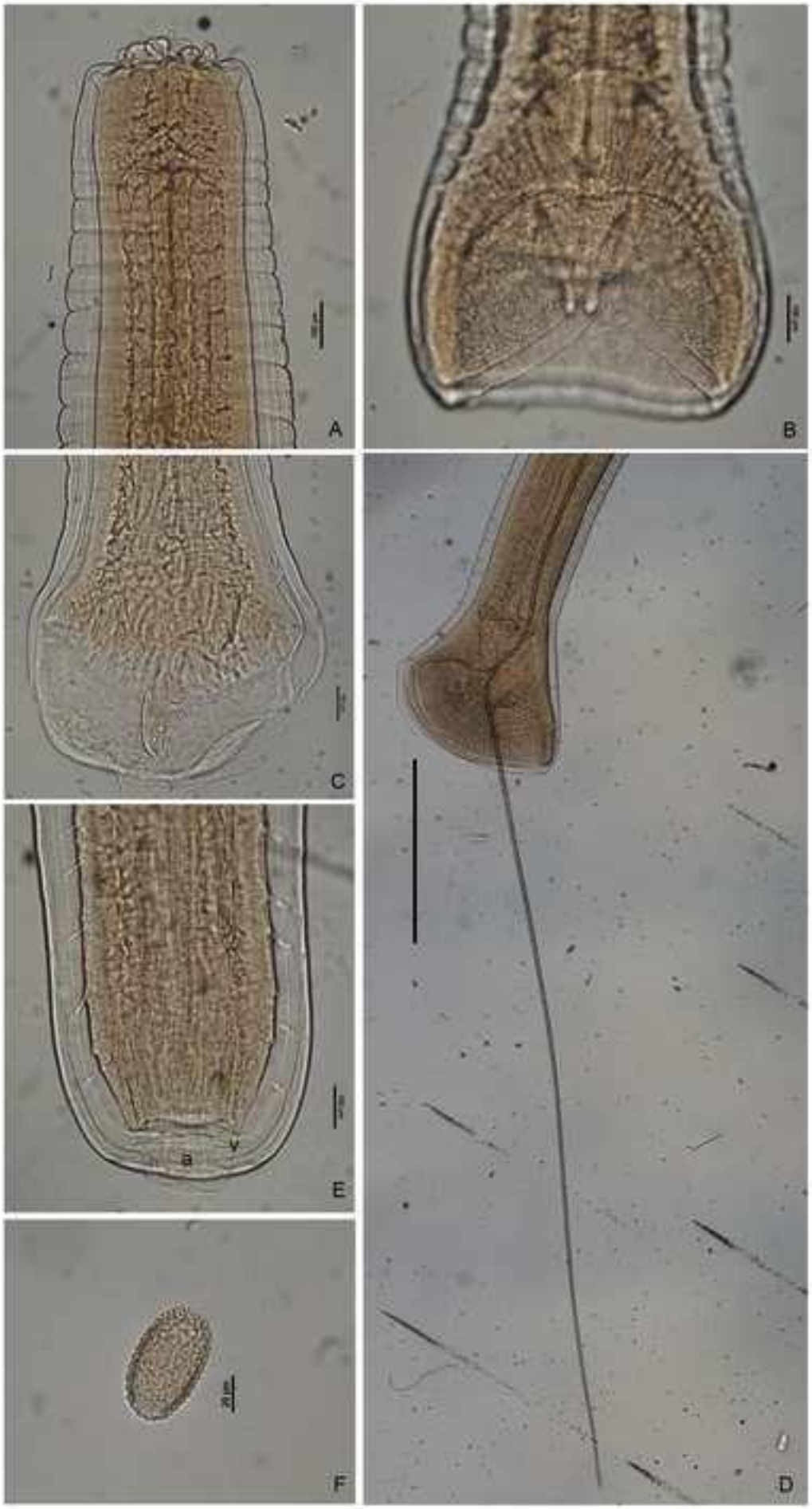
A
B
C
D
E
V
F

Table 1

Click here to access/download;Table;19-44 marked Table I.docx

Table I. Morphological data of *Eustrongylides excisus* adult [range (mean ± SD)]. Abbreviations: H = height, W = width, L = length.

Inner circle papillae (H × W)*	14-37 × 26-63 (27 ± 6.32 × 39 ± 8.81)
Outer circle papillae (H × W)*	10-44 × 43 - 82 (24 ± 9.01 × 66 ± 12.5)
Length of oral cavity*	97-193 (136 ± 28.63)
Width at nerve ring*	306-511 (400 ± 72.93)
Nerve ring from anterior extremity*	176-268 (223 ± 29.71)
Male caudal bursa (L × W)†	334-987 × 723-1726 (478 ± 167.83 × 949 ± 245.81)
Spicule (n = 4) length†	3,863-6,352 (4,752 ± 1,106.38)
Female width at caudal end‡	428-760 (667 ± 112.81)
Eggs (L × W)	67-69 × 38-42 (68 ± 0.97 × 40 ± 1.03)

* = 31 specimens.

† = 12 males.

‡ = 9 females.

Figure 2

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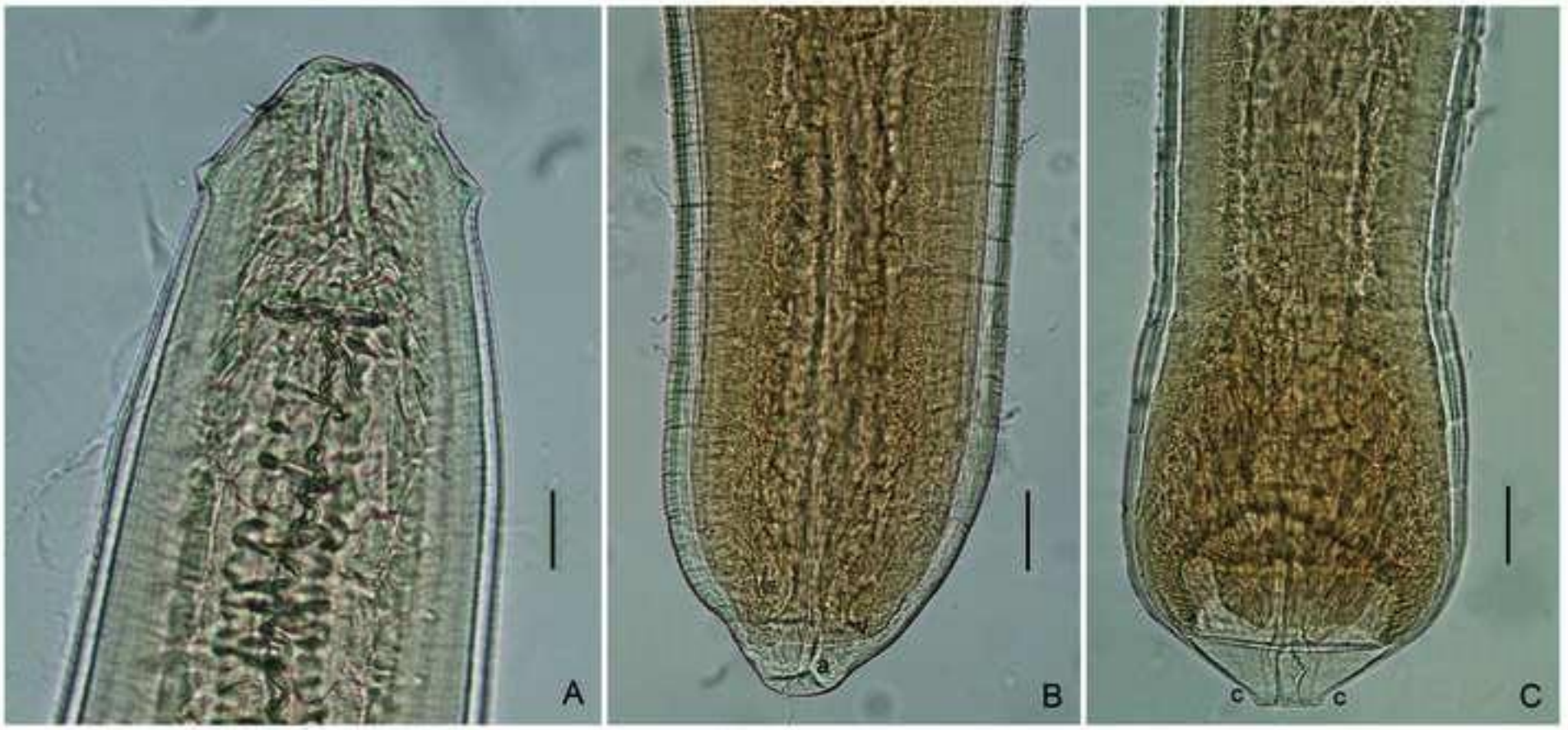

Table 2

Click here to access/download;Table;19-44 Table II.docx

Table II. Morphological data of *Eustrongylides excisus* fourth stage larvae [Range (mean ± SD)].

Total length	28,451-41,456 (34,829 ± 4,418.50)
Length of oral cavity	78-144 (107 ± 23.76)
Width at nerve ring	192-244 (216 ± 17.40)
Nerve ring from anterior extremity	142-177 (160 ± 13.47)
Esophagus length	8198-12,014 (9,817 ± 1,457.63)
Width at caudal end	359-441 (398 ± 29.24)

Figure 3

Click here to access/download;Figure;Fig. 3.tif

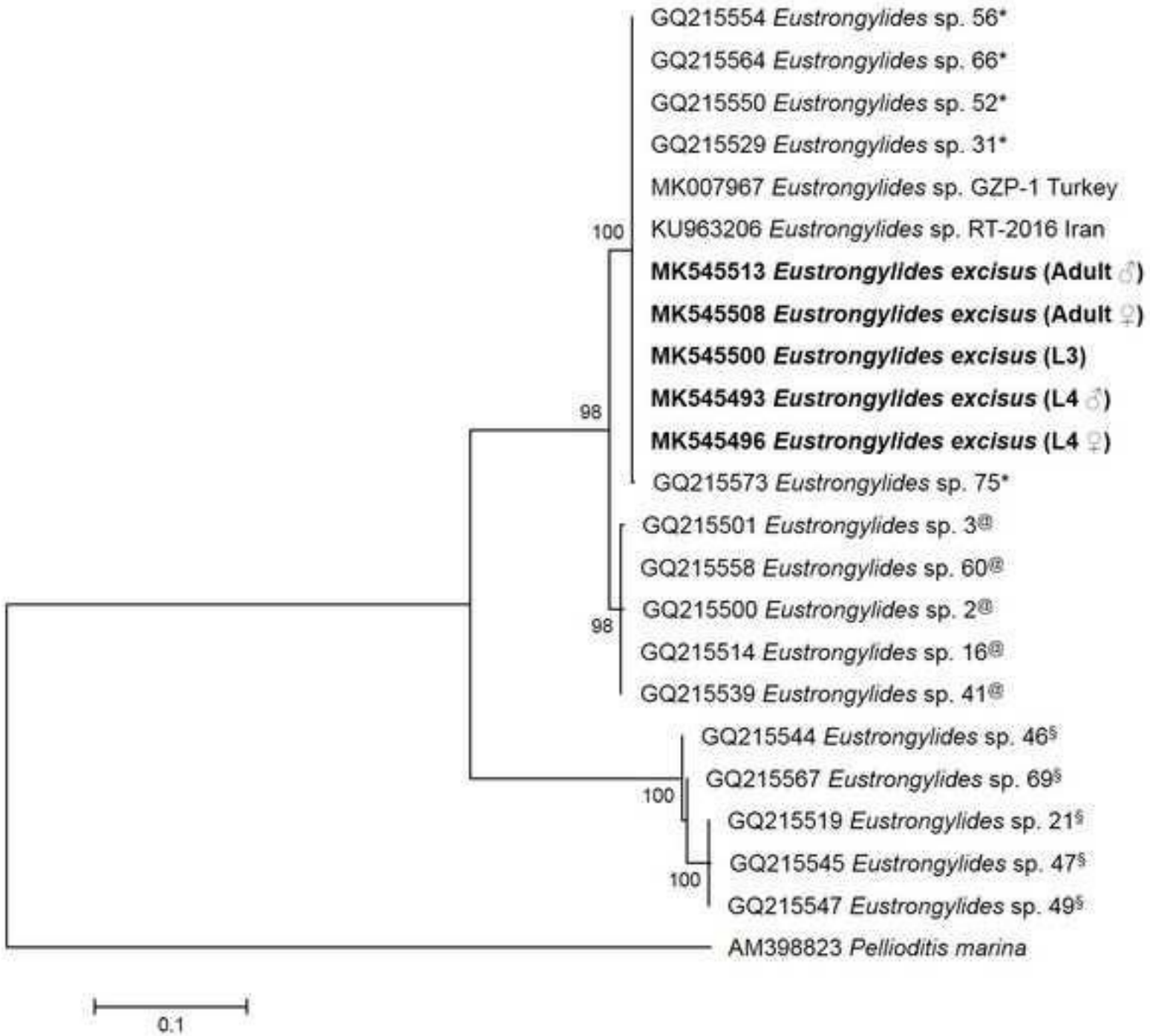
GQ215554 Eustrongylides sp. 56*
GQ215564 Eustrongylides sp. 66*
GQ215550 Eustrongylides sp. 52*
GQ215529 Eustrongylides sp. 31*
MK007967 Eustrongylides sp. GZP-1 Turkey
100
KU963206 Eustrongylides sp. RT-2016 Iran
MK545513 Eustrongylides excisus (Adult ♂)
MK545508 Eustrongylides excisus (Adult ♀)
MK545500 Eustrongylides excisus (L3)
98
MK545493 Eustrongylides excisus (L4 ♂)
MK545496 Eustrongylides excisus (L4 ♀)
GQ215573 Eustrongylides sp. 75*
GQ215501 Eustrongylides sp. 3@
GQ215558 Eustrongylides sp. 60@
98
GQ215500 Eustrongylides sp. 2@
GQ215514 Eustrongylides sp. 16@
GQ215539 Eustrongylides sp. 41@
GQ215544 Eustrongylides sp. 46§
GQ215567 Eustrongylides sp. 69§
100
GQ215519 Eustrongylides sp. 21§
GQ215545 Eustrongylides sp. 47§
100
GQ215547 Eustrongylides sp. 49§
AM398823 Pellioditis marina
0.1

Figure 4

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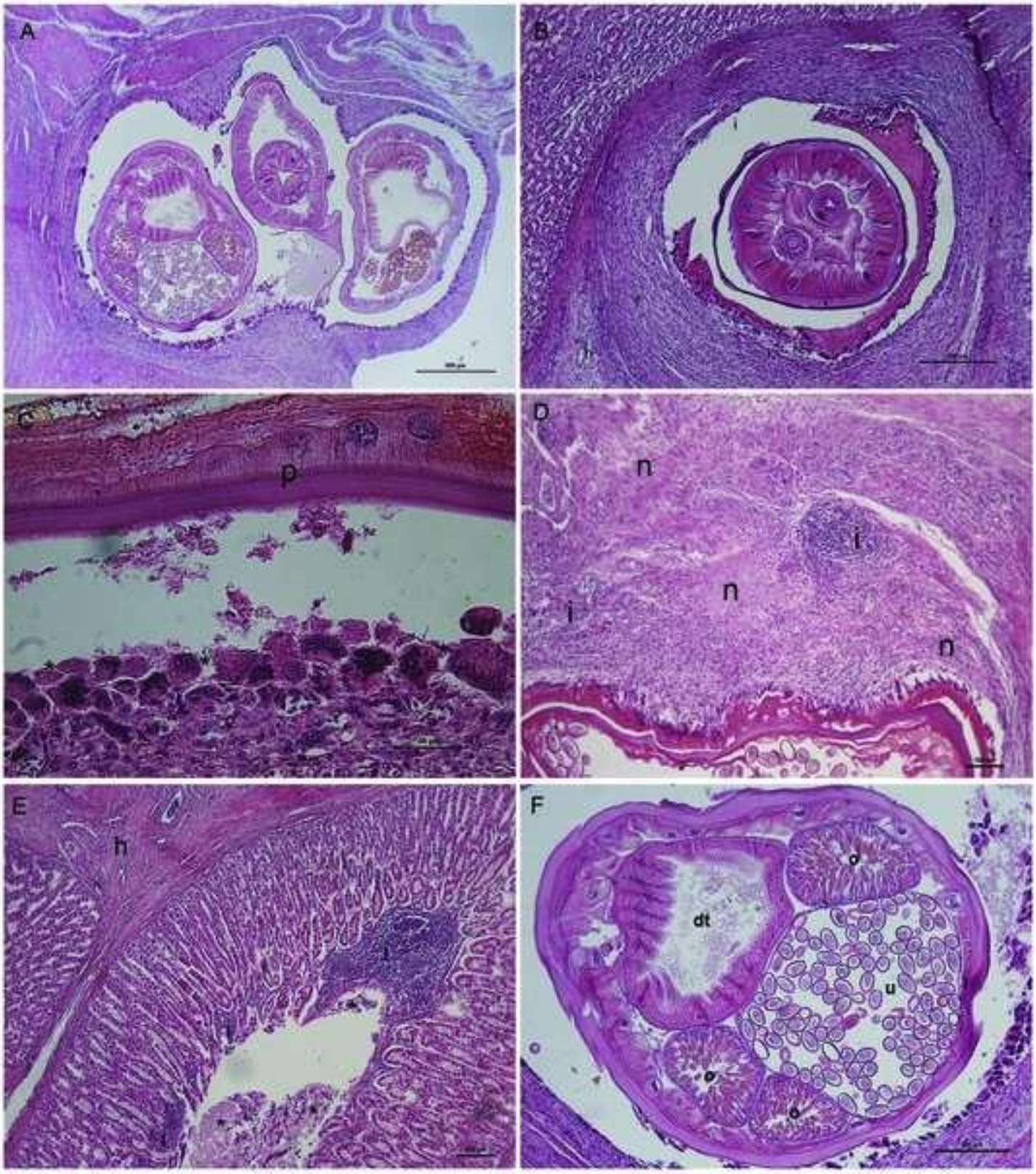

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Multiplex PCR for simultaneous identification of the most common European Opisthorchiid and Heterophyid in fish or fish products

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ABSTRACT

Among others, the families Opisthorchiidae and Heterophyidae includes several genera causing fish-borne zoonoses and distributed also in European Countries and that are included in the ParaFishControl (Advanced Tools and Research Strategies for Parasite Control in European farmed fish) H2020 EU project. Due to the small size of the metacercariae, the infective stage for human, these parasites cannot be detected visually in fish and monitoring requires expert application of time-consuming techniques. The aim of this was to develop a rapid and affordable molecular method based on multiplex PCR for simultaneous identification of metacercariae of the most common European Opisthorchiid and Heterophyid in fish or fish products.

1. Introduction

Fish-borne zoonotic trematodes are commonly reported worldwide, and >70 species are known to be zoonotic (WHO, 2011). Among others, the families Opisthorchiidae and Heterophyidae include several genera of zoonotic interest distributed also in European Countries. The life cycle of these parasites involves a snail as first intermediate host, fish as second intermediate host, mammals, including man, and fish-eating birds as definitive hosts (Chai et al., 2007). Humans and other definitive hosts can become infected by eating raw or undercooked fish or fish product containing infective metacercariae (Chai et al., 2007; Phan et al., 2011).

Members of the family Opisthorchiidae mature mainly in the hepatobiliary system of many piscivorous mammals and birds (Lim et al., 2008; Marcos et al., 2008). It includes 33 genera (King and Scholz, 2001) among which 3 species belonging to 2 genera are of major zoonotic importance such as *Opisthorchis felineus* (Rivolta, 1884), *O. viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Cobbold, 1875) (the last two not present in Europe) and some considered as minor zoonotic agents, *Metorchis* spp. (*M. bilis* Braun, 1890) and *Pseudamphistomum truncatum* (Rudolphi, 1819), both described in Eurasia (Sherrard-Smith et al., 2009; Mordvinov et al., 2012). The family Heterophyidae includes trematodes infecting vertebrate animals, including mammals and birds. At least 36 genera are known within this family (Pearson, 2008), with at least 13 genera and 29 species known to be zoonotic (Chai and Jung, 2017), among which *Metagonimus* spp. and *Apophallus* spp. have been reported in Europe.

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Transmission to humans occurs through the consumption of raw or undercooked fish muscle infected by the larval stage of the parasite, the metacercaria. Since the morphological approach poses some issues in identifying the species at this stage and discriminating zoonotic and non-zoonotic metacercariae, molecular techniques are necessary for their identification.

Due to the small size of the metacercariae (generally <400 μm), these parasites cannot be visually detected in fish and monitoring requires expert application of time-consuming techniques. The standard procedure, reported by the European Union Reference Laboratory for Parasites (ISS, Rome) to detect opisthorchiid/heterophyid metacercariae in fish fillets primarily involves artificial digestion of fish tissue with hydrochloric-pepsin or the compression of muscle between two-glass slides and observation under a stereomicroscope. Isolated metacercariae are then identified to genus level using morphological features, but they are very often difficult to distinguish due to the high morphological similarity.

In the framework of the EU project ParaFishControl (H2020, Advanced Tools and Research Strategies for Parasite Control in European farmed fish) the aim of this work was to develop a rapid and affordable molecular method for simultaneous identification of metacercariae of the most common European opisthorchiid and heterophyid in fish or fish products.

2. Materials and methods

For the design of the multiplex PCR, cercariae or metacercariae of opisthorchiid/heterophyid were collected. In details, opisthorchiid metacercariae of *Opisthorchis felineus* were isolated from the muscle of wild tench (*Tinca tinca*) from Bolsena Lake, central Italy and *Pseudamphistomum truncatum* metacercariae from wild tench sampled at Como Lake, northern Italy, while *Metorchis* spp. cercariae were collected from gastropods sampled from Iseo Lake, northern Italy. Heterophyid metacercariae of *Metagonimus* spp. were collected from *Alburnus alburnus* and *Chondrostoma nasus* while metacercariae of *Apophallus* spp., from *C. nasus*, *Abramis brama*, *Blicca bjoerkna*, *Gymnocephalus cernua*, *Perca fluviatilis*, *Scardinius erythopthalmus* and *Esox lucius* sampled in Hungary.

From all the developmental stages the DNA was extracted using Chelex100 (Sigma-Aldrich, Saint Louis, MO, USA) at 5% concentration. Briefly, 300 μl of 5% Chelex100 were added to 1 cercaria or metacercaria, incubated in heat block at 95 °C for 5 min. and centrifuged at full speed for 5 min. We run incubations from 5 to 20 min. and because we observed no differences, selected the shortest one. The supernatant containing the DNA was transferred into a clean tube and diluted at least at 1:10 for downstream use.

Three molecular markers were selected, 18S and ITS rDNA, and COI mtDNA and amplified by PCR with the primers reported in Table 1. The products were resolved on a 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5× TBE (Invitrogen – Thermo Fisher Scientific, Carlsbad, CA, USA). For sequencing, bands were excised and purified by NucleoSpin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany) and sequenced in both direction with an ABI 3730 DNA analyzer at StarSEQ GmbH (Mainz, Germany). The sequences were assembled with Vector NTI Advance™ 11 software (Thermo Fisher Scientific, Carlsbad, CA, USA). In order to confirm their identity, the sequence of each molecular marker was firstly subjected to BLAST search and then aligned using ClustalW included in Bioedit software and adjusted by eye.

Multiplex PCR primers were designed manually or using Primer-BLAST and then checked for primer-dimers with the web tool Multiple Primers Analyzer (Thermo Fisher Scientific). The primers were chosen basing on different fragments size in order to distinguish *O. felineus*, *P. truncatum*, *Metorchis* spp., *Apophallus* spp. and *Metagonimus* spp.

3. Results

All the molecular markers for all the five genera/species under consideration were successfully amplified and sequenced.

The sequences of the 18S rDNA gene was ~1860 bp; however, the multiple alignment showed no possibility to find a regions variable enough which could be used to design primers, as it resulted to be highly conserved (overall distance 0.01). For this reason, it was not further considered. The sequences of the entire ITS rDNA was ~1600 bp (including the end of 18S and the beginning of 28S). The multiple alignment showed the presence of variable regions in which some of the primers were designed. A common reverse primer (comm_r) was designed at the beginning of the 28S rDNA, and genus specific primers were designed for *Apophallus* spp. (~1066 bp) and for *Metagonimus* spp. (~722 bp). The ITS rDNA primer set yielded multiple amplifications among genera in *O. felineus*, *Metorchis* spp. and *P. truncatum*, and a COI mtDNA primer set was therefore developed. The length

Table 1
Primers used for the amplifications of the 18S and ITS rDNA and COI mtDNA.

	Gene	References
82_f - CAGTAGTCATATGCTTGTCTCAG	18S rRNA	Mariaux, 1998
81_r - TTCACCTACGGAAACCTTGTTACG		
83_f - GATACCGTCCTAGTTCTGACCA		
84_r - TCCTTTAAGTTTCAGCTT GC		
81_f - GTAACAAGGTTTCCGTAGGTGAA	ITS rRNA	Gustinelli et al., 2010
ITS2_r - CCTGGTTAGTTTCTTTTCCTCCGC		Cribb et al., 1998
Dice1F ATTAACCCTCACTAAATTWCNTTRGATCATAAC[a]	COI mtDNA	Van Steenkiste et al., 2015
Dice11R TAATACGACTCACTATAGCWGWACHAAATTTHCGATC		

[a] Shortened T3 and T7 tails at the 5′ end of Dice1F and Dice11R, are underlined and were used for sequencing.

Table 2
Primers designed and tested in the present study.

		Gene	Product
Ap4_f	CCAAGCGAAATCTTCCAAGG	ITS rRNA	~1066 bp
Mt3_f	TTGAATAATGTGACACGAGC		~722 bp
comm_r	ATATGCTTAAGTTCAGCGGG		
Me4_f	GTGGGTTTTTAGGACTTGGG	COI mtDNA	~500 bp
Me4_r	ACCTGCTGCCAACACAGGTAA		
Ps1_f	CCTCTCCTGTTAGGGTTGTC		~150 bp
Ps1_r	CCCGATGACAGGGGAGGATA		
OF9_f	TGTGAGGCGGGTTTACAGGA		~230 bp (Caffara et al., 2017)
OF6_r	CTCCAGCCCCACCATACATT		

Ap = *Apophallus*, Mt = *Metagonimus*, Me = *Metorchis*, Ps = *Pseudamphistomum*, OF = *Opisthorchis felineus*, comm_r = common reverse.

of the COI mtDNA sequence was ~640 bp. In this gene the high variability allowed design of primers for *Metorchis* spp. (~500 bp) and *P. truncatum* (~150 bp). Finally, for *O. felineus* the species-specific primers designed previously by Caffara et al. (2017) were used (~230 bp). In Table 2 the list of the final set of primers designed and tested is reported.

The primer set was tested firstly in singlet PCR of 25 μl, with 2× DreamTaq Hot Start Green PCR Master Mix (Thermo Scientific, Thermo Fisher Scientific, Carlsbad, CA, USA) containing 2× DreamTaq buffer, dNTP's (0.4 mM of each), 4 mM $MgCl_2$, 0.3 μM of each primer and 2.5 μl of template DNA. The thermal cycler program (Tpersonal, Biometra) was 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, preceded by a denaturation step at 95 °C for 3 min and followed by an extended elongation step at 72 °C for 10 min. The products were resolved in 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5× TBE. All the primer sets successfully amplified products of the expected size.

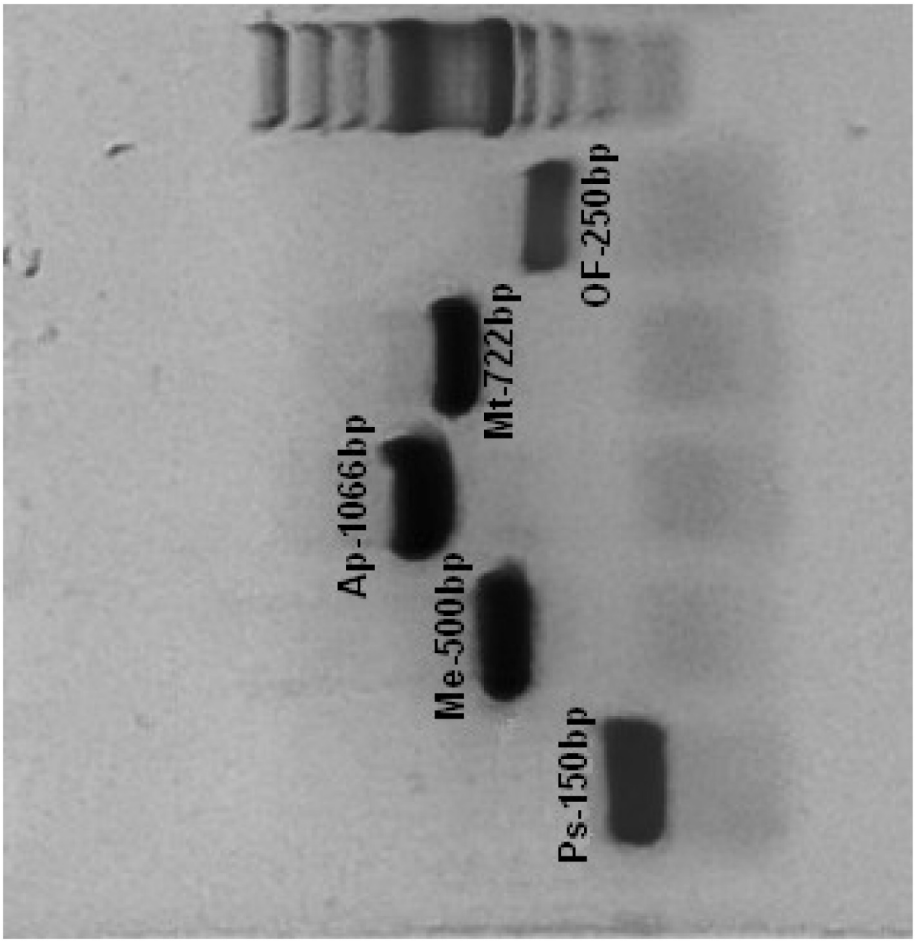

Fig. 1. Multiplex-PCR products of *Pseudamphistomum truncatum* (Ps), *Metorchis* spp. (Me), *Apophallus* spp. (Ap), *Metagonimus* spp. (Mt) and *Opisthorchis felineus* (OF). Molecular marker 100 bp.

The primers were then combined in multiplex PCR using the same protocol as singlet PCR. Different primers concentrations were tested: 0.25 μM, 0.2 μM and 0.1 μM. The concentration giving the best results was 0.2 μM. The combined primers successfully amplified the expected products, even if some aspecific bands were present. To evaluate the specificity of these primer set and the conditions of aspecific binding, a PCR gradient using Veriti® 96-Well Thermal Cycler (Applied Biosystem) was performed twice. The annealing temperatures tested were from 51 °C to 56 °C and from 57 °C to 62 °C. An annealing temperature of 60 °C gave the highest specific band intensity without non-specific products (Fig. 1). Finally, all the genus/species specific bands obtained were excised, purified and sequenced (see Materials and Methods) in order to confirm their belonging to the genus/species. The BLAST results confirmed the identity of *O. felineus* (EU921260), *P. truncatum* (KP869085), *Metorchis* spp. (KY075779), *Metagonimus* spp. (MF407173) and *Apophallus* spp. (MF438057 *A. donicus* and MF438066 *A. muehlingi*).

4. Discussion

A central prerequisite for control of food-borne zoonotic trematodes is an accurate diagnosis at least at genus level. Freshwater fish muscles can harbor metacercariae of species that differ in zoonotic importance and that cannot be distinguished with conventional light microscopy. The multiplex PCR developed in the present study may provide a rapid and cost-effective tool for the simultaneous identification of *O. felineus*, *P. truncatum*, *Metorchis* spp., *Metagonimus* spp. and *Apophallus* spp. the main zoonotic or potentially zoonotic trematodes present in Europe. Moreover, this multiplex PCR could be used to identify the abovementioned genus/species at any developmental stages (eggs, cercariae, metacercariae and adult). Currently, the identification of these genera/species is based on PCR followed by sequencing, a time-consuming and expensive technique, except for *O. felineus* for which a species-specific primer has been published previously by Caffara et al. (2017). Multiplex PCR has been successfully developed for other zoonotic parasites or their hosts, such as for example *C. sinensis* and *O. viverrini* (Le et al., 2006), for distinguish among *Galba* species, host of *Fasciola hepatica* (Alda et al., 2018), or simultaneous detection of *Schistosoma mansoni* and the *Biomphalaria* (Jannotti-Passos et al., 2006). In conclusion the multiplex PCR herein developed is an accurate and affordable method that avoids the costs of sequencing and enables rapid identification of any developmental stage of five potentially zoonotic agents in a single reaction.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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10. Conclusions

During the last decades of the Twentieth century, global aquaculture has experienced increasing trends in terms of productions volume and turnover, with interannual growth rates of around 6% to 10%. However, during the last decade global aquaculture has known a marked contraction of this growing trend and European aquaculture has been walking the same path, since aquaculture production in almost all European countries has stagnated for more than ten years (FAO, 2020). FAO last reports identified several factors contributing to this worrying slowdown. Amongst factors most contributing to this significant decrease, experts listed outbreaks of transmissible diseases, whose negative effects on aquaculture productivity have been estimated for decades.

Compared with other pathogens, such as viruses and bacteria, parasites have long been a neglected group of pathogens, and this continues to be their position today (Robertson, 2018). Nevertheless, parasitic diseases attributable to obligate or opportunistic eukaryotic pathogens continue to have a major impact on global finfish and shellfish aquaculture, and in many regions, they represent a key constraint to production, sustainability, and economic viability of aquaculture (Shinn *et al.*, 2015). Parasites impact on mariculture productions is closely related to intensive aquaculture management procedures. In wild populations, parasites usually have an aggregate distribution, with low parasitic burden on few animals and most of the susceptible population free from parasites. This peculiar aspect of parasites behaviour within wild animal populations leads to an ecological coexistence between parasites and host populations, with extremely low mortality and morbidity rates. Intensive fish farming practices, by maintaining large numbers of fish confined in a small area, create conditions for an increased risk of parasitic disease outbreaks, by providing higher density of fish, repeated introduction of naive hosts, homogeneous host populations, fast growth, and a potential decrease in genetic diversity (Nowak, 2007). All these factors make parasitic diseases an emerging issue for global aquaculture.

Gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) represent the most farmed marine fish species in the Mediterranean area, nevertheless updated information on the occurrence of emerging parasitic diseases within sea bream and sea bass aquaculture is lacking. Although *Cryptosporidium molnari* and *Enterospora nucleophila* presence in gilthead sea bream husbandry has been reported sporadically for twenty years, they are still considered as “emerging parasites” in Mediterranean aquaculture. Deficiency of consistent data on these two enteric parasites is mainly due to the significant challenges in detecting their presence within culture systems, even when clinical signs are noticeable among affected fish populations. The purpose of this work was to map and deepen the knowledge on *C. molnari* and *E. nucleophila* occurrence in Mediterranean aquaculture. Thus, the research activities presented in this work primarily focused on development and validation of affordable and effective diagnostic techniques, based on molecular technologies. The application of these innovative tools to the study of *E. nucleophila* and *C. molnari* enabled the

achievement of the first consistent data on their occurrence in gilthead sea bream husbandry across the Mediterranean area.

In analysing specimens collected in a considerable number of farming sites, this work gave an overall picture of a wide but non-homogeneous distribution of *E. nucleophila* and *C. molnari* in the Mediterranean Basin within different production phases and types of facility.

E. nucleophila and *C. molnari* were detected in almost half of the surveyed farms (41% and 47% respectively) and they were simultaneously present in nearly one third of the facilities. Moreover, data concerning the presence of the parasites at different rearing stages and at different rearing conditions highlighted a non-homogeneous distribution. Parasites were mainly detected in post-weaning steps of production, with *E. nucleophila* higher detection at ongrowing and sea-cages as type of rearing system, and *C. molnari* higher positivity percentages at pre-growing and ponds as type of rearing. Moreover, univariate analysis underlined a statistically significant association between *C. molnari* detection and pre-growing production step and tanks as a rearing system. The absence of positive eggs or positive samples at early production stages (larval and post-larval sector) led to consider vertical transmission extremely unlikely (albeit demonstrated for some microsporidia), and to confirm the role of water in the transmission and maintenance of the infection. As concerning *E. nucleophila*, the incomplete knowledge about the biological cycle of the parasite does not allow to rule out the existence of a hypothetical wild reservoir, especially for the maintenance of the infection in marine grow-out systems.

The use of a risk factors individuation-based method for analysing data provided interesting findings partially explaining parasites' distribution patterns within different production stages and systems, which may be studied in depth in future. Further studies may be targeted at exploring risk factors for the detection of the two parasites which have been individuated so far, and they may strive to assess the pathological and economic impact of *E. nucleophila* and *C. molnari* in gilthead sea bream aquaculture, with a particular focus on production phases highlighted by this work.

As regarding fish-borne zoonotic parasites, they have been public health orphans in the world of research funding, due in no small measure to insufficient appreciation of the crucial fact that their transmission is often dependent on well-entrenched human behaviours (Chai *et al.*, 2005). Since most fish-borne parasites share transmission routes to humans, these zoonoses may have a much greater aggregate effect than some other better-known parasitic diseases. The challenging diagnosis, the complexities of human cultural behaviours and the poor understanding of potential economic costs have made this field simultaneously daunting, scientifically obscure and, therefore, somewhat unattractive to investigators especially in developed countries (Chai *et al.*, 2005).

Globalisation contributes to expose an increasing portion of the world's population to a wider range of foodborne parasites (Robertson *et al*, 2014). Globalisation has opened new markets and it has transformed food consumption patterns, resulting in rapid and massive cultural change. Globalisation have also facilitated the spread of

transmissible diseases, especially foodborne parasitic zoonoses, and this can have enormous negative consequences on food security, food safety, and food sovereignty. In addition to health effects, globalisation of foodborne parasites may also have serious economic consequences (Robertson *et al*, 2014).

The emergence and re-emergence of fish-borne zoonotic parasitic diseases is a global phenomenon which has also been well documented in EU countries. The research activities reported in this work aimed at shedding light on the presence of emerging parasites in fishery products from European freshwater environments. The work on *Eustrongylides excisus* provided the first consistent information on the nematode's identity and epidemiology in Central Italy lakes and confirmed the presence of the parasite's infective stages within the flesh of edible freshwater fish, thus confirming its potential to affect consumers of fish products from local markets. Moreover, the work provided a reliable molecular assay for the identification of parasite's larvae collected from infected fish.

The research activities in the field of fish-borne zoonoses which are reported in this work also contributed to the development of a novel molecular diagnostic technique for the detection of zoonotic trematodes within freshwater fish products. The multiplex PCR assay which has been developed in the reported study may provide a rapid and cost-effective tool for the simultaneous identification of *Opisthorchis felineus*, *Pseudamphistomum truncatum*, *Metorchis* spp., *Metagonimus* spp., and *Apophallus* spp., the main zoonotic or potentially zoonotic trematodes circulating in Europe. Since the identification of most of these parasites currently relies on genus sequencing, the developed multiplex PCR assay provides a rapid, accurate, and affordable method which avoids sequencing costs and enables the rapid identification of any developmental stage of five potentially zoonotic pathogens in a single reaction.

Facing the challenges brought about by the evaluation of novel diagnostic assays for fish parasites, this work has underlined the need for continuous efforts in developing increasingly sensitive and specific diagnostic techniques.

As regarding gilthead sea bream aquaculture, the impossibility to detect *E. nucleophila* and *C. molnari* by routinely fish examination at farming sites and the unsuitability of traditional diagnostic procedures to serve this purpose have determined the neglection of the two diseases for over a decade. The development of effective and easy-to-use molecular diagnostic tools is therefore a major requisite for understanding and describing parasites' distribution patterns in gilthead sea bream husbandry. The combined use of a risk individuation-based approach to data analysis can lead the way to a wider and shared use of a risk factors evaluation approach to transmissible diseases in Mediterranean aquaculture, as it is already systematically employed in the management of Scandinavian aquaculture. Thus, innovative investigation methods represent a fundamental requirement to set guidelines drawn up by international institutions aimed at standardizing surveillance plans in the Mediterranean Sea. EU institutions commitment to a risk assessment-based organization of animal husbandry is strongly embodied by the new regulation on animal health and control of transmissible diseases (Regulation (EU) 2016/429 of the European Parliament). The handling of new issues

arousing from emerging transmissible diseases in aquaculture systems, which has been proposed in this work, may represent the way to face the demanding requests of both EU institution and aquaculture stakeholders.

At the same time, the development of novel diagnostic techniques for the detection of fish-borne zoonotic parasites is a critical requirement to deepen our understanding of parasites distribution patterns and epidemiology within fish populations and consequently to update the current regulation on food safety for fishery products.

Over the past decades, aquatic animals' parasitic diseases have shown their potential to spread rapidly, irrespective of national borders. The Mediterranean aquaculture and fishery industries are highly connected either geographically or through trade routes, therefore addressing shared measures for controlling parasitic diseases is of paramount importance. Next years' challenge is implementing aquaculture sustainability and proficiency by preventing and reducing the impact of parasitic diseases.

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