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Emerging infections in the Mediterranean mussel *Mytilus galloprovincialis* in Campania Region (Italy)

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Title

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Abstract

Emerging Infectious Diseases (EIDs) have been reported affecting several marine organisms in the past years however EIDs of invertebrates have to date been poorly described, due in part to a lack of interest and no expert in the field. Historical data have shown the constant presence of undescribed pathogens belonging to the group of microsporidia and trematode in *M. galloprovincialis* in Campania Region, although scarce are the information in literature about their emergence and effect on the host.

Microsporidia are a spore-forming, obligate, intracellular parasites infecting all major taxa in all environments. Of the 187 genera described, almost half are known to infect aquatic organisms, mainly teleost and crustaceans. In molluscs main descriptions are related to one genus (*Steinhausia* sp.) reported in bivalves' and gastropods. The species *S. mytilovum* and *S. ovicola* affect bivalves belong to the families Mytilidae and Ostreidae, with a *Steinhausia*-like parasite being described in the cockle *Cerastoderma edule*. Major species descriptions include detailed microscopic and ultrastructural images, but no information is present about pathogen phylogeny. In this study we report the constant occurrence of the microsporidian parasite *S. mytilovum* affecting the population of mussel, *M. galloprovincialis*, in one natural bed and 13 farms along Campania region coastline (Italy). *S. mytilovum* affected 9 out of the 13 farms, with a prevalence ranging from 2-30% of the females, depending on the areas and the seasons. The parasite develops at oocyte level within a sporophorous vesicle where it produces a spore in a number of one, three per cell, at cytoplasm and at nuclear level. *S. mytilovum* elicited an infiltrative (24,8%) or a strong capsular inflammatory response (43,4%) at gonadal follicles and surrounding vesicular connective tissue, in some case accompanied by gonadal atresia (24,8%), eventually associated to loss of gonadal architecture. In other cases (7%) no reaction was observed. Microsporidian systematics currently turn around five taxonomic clades, identified by SSU ribosomal gene sequence data. Neighbor Joining of the 18 S assigned *S. mytilovum* in a separated branch within the Clade V, defined as the Class Terresporidia, with

closest genetic relationship (84% identity) to an undermined invertebrate's ovarian microsporidian.

The second part of the study have identified the systematic presence of metacercariae belonging to the group of helminth trematodes at the foot level of 323 examined mussels.

Trematodes can have a variety of target tissues, which can result in serious damage in the mollusc and major economic loss. The metacercaria phase of trematodes infections in bivalves is the most well-documented and affects several organs and tissues, particularly the gonads and gills. Recently, many mussels have shown the presence of inflammatory lesions at the foot level linked to the presence of trematodes metacercariae or unidentified amorphous structures.

To date, there is a single report of trematodes with the same tropism, in particular in Atlantic mussels (*M. edulis*) in the waters of Great Britain and Ireland and reported to belong to the group of Echinostomates digenea, and to the species *Echinostephilla patellae*.

The characterization of the parasites has been carried first with a macroscopic identification, the mussels' foot was separated and cut in half, revealing occasionally the presence of an encysted parasite. Light microscopy has shown the presence of metacercariae in mussels' foot in all the sampled areas in 27 out of 323 animals, in a number 1-5 per animal, with a prevalence ranging from 3,3% to 16,67%, highest prevalence was observed in warm months. The presence of the parasite resulted in three different type of lesions: inflammatory reaction associated both to metacercariae and cercariae and nodular lesions. Molecular diagnostic carried out on the 18 S rDNA showed an identity of 94.2% in genebank with digenean trematodes of the genus *Opisthomonorcheides* spp.

CHAPTER 1: Introduction

1. Emerging Infectious Diseases (EIDs) in the marine environment

Infectious diseases are an important element in an ecosystem equilibrium. Infections affect people, domestic animals and wildlife, with many pathogens being able to infect multiple species.

The so called Emerging Infectious Diseases (EIDs) have been defined as "infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range" (Morse, 1995). To better understand the dynamics of the emergence of those infections a further classification could be implemented: EIDs could be classified as "newly emerging", "re-emerging/resurging", and "deliberately emerging" (Morens, 2004).

The emergence of these diseases, and resurgence of old ones is the result of various factors, including changes in pathogens (switching from animal to human host, genetic mutation) and changes in hosts or intermediate vectors. An important role in to emergence has been correlated with human behavioural changes and human-induced global changes (Weiss, 2004).

In the past years emerging diseases outbreaks have been reported affecting marine organisms: cetacean, pinniped, and fish populations have been affected, often severely, by algal toxins and/or viral epidemics (Epstein, 1998) brought to raise the attention of researchers worldwide on the emergence of diseases in marine species. Mass mortality events particularly along heavily polluted coastal areas, suggest human activity as a factor in disease dynamics (Harvell, 1999) but also impacted marine ecosystems such as in seagrasses (Burge, 2013), oysters (Mann, 2009), and sea urchins (Lauzon-Guay, 2009). Climate variability and change and human activity can play a key role in epidemics compromising host resistance and immune response and facilitating the pathogen transmission. (Harvell, 1999)

Environmental changes could facilitate the emergence of a new disease or increase the prevalence and virulence of an existing pathogen (Harvell, 2002). Climate-induced changes along with the current trend of a warming climate impacts on

marine host-pathogen-environment equilibrium increasing the expression of on infectious diseases in marine invertebrate (corals, abalones, and oysters) and vertebrate (marine mammals, finfish, and humans) species (Burge, 2014).

The El Nino Southern Oscillation (ENSO) is one of the more visible climate variations that has had large-scale effects on marine ecosystems. In the past the ENSO events occurred with a frequency of 1 or 2 per decade but the impact of climate change has increased its occurrence with strongly related effects such as coral bleaching that caused mass mortality worldwide due to long term exposure to high water temperatures (Bouma, 1997). ENSO events have been implicated in spread and outbreaks of the protozoan parasite *Perkinsus marinus* and *Haplosporidium nelsoni* in oysters throughout the Gulf of Mexico, infection intensity closely follows the ENSO cycle. (Powell, 1992; Ford, 1996; Dekshenieks, 2000).

Human activity has greatly enhanced global transport of marine species including pathogens, and, it has been suggested that most mass mortalities of bivalve molluscs have resulted from transfer of infectious stocks.

2. Monitoring bivalve diseases in aquaculture

Over the past 50 years, the aquaculture industry has grown faster than any other food-producing sector, and it is estimated that by 2050, aquatic production will need to reach almost double its current level in order to supply global demand (Bostock et al., 2010; Stentiford et al., 2012). Marine invertebrates play an important role in the growing aquaculture industry, especially species such as oysters and shrimp, and these species are affected by emerging diseases associated with human-related activities such as movement of infected animals and culture-related practices. Warming seawater temperature brought up emergence of pathogens causing food-born illness such as noroviruses or vibriosis which remains a concern in sanitation of aquacultural shellfish, furthermore infectious diseases of the cultured animals themselves are perhaps the most serious threat to marine mollusc production. (Groner, 2016). As we cited previously severe outbreaks have brought significant economic destruction: for instance, epizootics of protistan parasites *Haplosporidium nelsoni* and *Perkinsus marinus* in the US Atlantic coast (Andrews,

1964; Haskin, 1957; Burreson, 1988) and emergence of *Marteilia refringens* (Comps, 1970) and *Bonamia ostreae* (Pichot, 1979) in Europe greatly reduced oyster populations and the industries that depend on them. Outbreaks of herpesviruses have affected abalone populations and related industries in Taiwan and Australia (Hooper, 2007), and emergence of 'microvariants' of herpesvirus OsHV-1 in *Crassostrea gigas* in Europe, Australia and New Zealand threatens global production of this aquaculture species. (Jenkins, 2013; Segarra, 2008). The physiology of the animals and the peculiarities of the culture system limit the efficacy of any treatment for diseases in molluscs (Berthe, 2008). Vaccinations are not possible in molluscs since they do not have an adaptive immune response. Also, the culture system is in an open environment, which makes field treatments challenging because of the need to consider the environment's impact. Because of this, therapeutic procedures have not been used in mollusc aquaculture, despite the fact that several illnesses and parasite treatments have proved effective in the lab or on a small scale in the field (Friedman, 2007). Rigorous procedures are applied to minimize the risk of introducing an infectious disease into a disease-free area. Monitoring potentially concerning pathogens is a major challenge for international aquaculture; rapid detection and prevention is needed to reduce the impact of epizootics (Lafferty, 2016). Standards, guidelines, diagnostic manuals and recommendations for diseases of economically important organisms are provided by the the World Organisation for Animal Health (WOAH) founded as OIE (Office International des Epizooties). OIE is an intergovernmental organization created by an International Agreement in 1924, responsible for improving animal health worldwide. the OIE established a list of significant diseases in order to promote effective control and prevent the spread of those diseases. At present 117 diseases are listed among which 7 are diseases of molluscs with the majority being represented by protozoans, one bacterial disease, infection of abalone with *Xenohaliotis californiensis*, and one viral disease, infection with abalone herpesvirus. The OIE has not legislative or executive functions, does not provide measures for animal health, but collects, analyses and disseminates information on outbreaks of certain diseases, those notifiable, to ensure the States that join the utmost transparency on the zoognostic situation about world diseases

in the listed OIE diseases. The listed diseases included in the law for bivalve molluscs are Marteliosis, Bonamiosis, Microcytosis and Perkinsosis. However, the use of notifiable pathogen lists can leave blind spots regarding the detection of unlisted and emerging pathogens; the number of EIDs is increasing because of globalization, urbanization and climate change and a special attention is given to the detection of newly recognized or suspected pathogens and known pathogens that may change their pathogenic potential or spread to a new geographical area or species. (Carnegie, 2016). In the European Union, since 9th of March 2016, is applied the new Animal Health Law, the Regulation (EU) 2016/429, that updates the previous directive 2006/88/EC requirements for aquaculture and products and on the prevention and control of certain diseases in aquatic animals. The new Animal Health Law focuses on early detection, prevention ed eradication of diseases, with the control of listed diseases and a major consideration on emerging diseases. (Part I, Chapter 2, art. 5-6). Since the production of molluscan aquaculture continuously increases more attention should be placed on monitoring health and diseases of those invertebrates, although as technologies and diagnostic assays are growing in veterinary medicine, there is a lack of interest on invertebrate pathology and pathogens (Braun, 2006; Carnegie, 2016).

3. Molluscan Bivalves diseases outbreak in the Mediterranean Sea

EIDs of invertebrates have to date been poorly described in the last years, due in part to a lack of interest and no expert in the field, this implied a lack of baseline data leading to a lack of specific and sensitive diagnostic tools for regular surveillance (Burge, 2016). Nevertheless, understanding infectious diseases affecting also non commercially important invertebrates has become a necessity due to outbreaks resulting in loss of biodiversity such as reef-building corals. Recent attention has been focused on climate change and other anthropogenic influences on infectious diseases, including those infecting invertebrate hosts. In fact, diseases infecting sessile marine invertebrates can be more easily correlated with climate change than their vertebrate host counterparts, such as finfish or marine mammals, in part due to the sedentary nature of most of their adult lifeforms (Burge, 2014). For many EIDs, documented changes in host–pathogen relation have been

linked to direct (e.g., movement of hosts) and indirect effects (e.g., climate change via CO_2 emissions) of human activities. Animal movement, often coupled with increased densities, is frequently cited as a major cause of disease emergence in domesticated invertebrates and in the natural populations of invertebrates they interact with; invasive hosts associated with climate change or habitat destruction can also play an important role in host pathogen shifts, shared resources such as food or substrates may facilitate the emergence from domestic to wild population through horizontal transmission. For example, in bivalve molluscs, disease transmission can be facilitated by filter-feeding (Ben-Horin, 2015) that occurs when infected individuals (often domesticated) share water resources with naive populations (domesticated or wild). Human activities that result in a compromised host population (immune-compromised, injured, or otherwise stressed) may also facilitate disease emergence, especially for ubiquitous, opportunistic pathogens (Burge, 2013). Like the movement of hosts, the movement of infected hosts, vectors/intermediate hosts, or contaminated equipment is also significant for disease emergence in invertebrates. Newly evolved pathogens are less reported in invertebrate EIDs, but mutation or gene-transfer events allow invertebrate pathogens to evade immune systems of hosts, increase host range, or acquire new virulence factors. As in the development of antibiotic resistance, human activities create environments that promote mutation or gene transfer among potential pathogens (Gillings, 2016).

Increasing temperature conditions due to anthropogenic climate change can affect the distribution of ubiquitous pathogens. Climate change directly impacts the homeostasis of an invertebrate and its pathogens: changes in temperature, pH, salinity, humidity, and precipitation patterns, can play an important role in disease emergence. increasing temperature conditions due to anthropogenic climate change can affect habitat range (temporally or spatially) or increase the virulence of pathogens and in particular bacterial pathogens. For example, warm winter conditions allowed the spread of the oyster pathogen *Perkinsus marinus* further north along the eastern coast of the United States (Ford, 1996; Cook, 1998), while increased sea-surface temperatures enhance the virulence of the coral pathogen *Vibrio shiloi*, so that coral bleaching at lower temperatures switches to into coral

mortality at high temperatures (Kushmaro, 1998). Organism health (and populations losses) and/or disease emergence may be impacted by anthropogenic stressors such as changes in climate, pollutants, habitat loss, and diet. The effect of a combination of multiple stressors (global or local) on disease dynamics is difficult to investigate, and therefore is under studied. Some of the best examples of EIDs outbreaks that significantly disrupted commercial ventures come from aquaculture or marine systems.

In the past decades episodes of mass mortalities and significant loss in bivalve's aquaculture has led the researchers to focus on the study of EIDs in those species. Regarding the Mediterranean basin several outbreaks were reported in commercial and wild species, both for introduction of diseased animals or seeds from outside the Mediterranean or for the interaction between existing pathogens and stressor factors such as increasing temperature due to climate change. Some pathogens are more likely to be reported as they are OIE listed, as in the case of *Perkinsus olseni*, other pathogens may be underestimated due to lack of monitoring but can lead potentially to emerging zoonosis, as in the case of *Nocardia crassostreae*. Wild populations have been affected by mass mortalities events, in the past years the population of the pen shell Pinna nobilis drastically declined after the outbreaks of multiple infections.

3.1 The dinoflagellate Perkinsus sp.

The genus *Perkinsus* includes parasites of marine molluscs and gastropods, this protistan parasite has been associated with mass mortalities events worldwide, for example the mass mortality of *C. virginica* in the USA (Ray, 1996). Due to the severe economic loss for fisheries and aquaculture the presence of this parasite has been attentioned making Perkinsosis an OIE-listed disease. Perkinsosis is described in numerous studies and reports as occurring in commercially significant molluscan species in Europe: *P. atlanticus* and *P. olseni,* once described as different species, are now recognized as unique (Murrell, 2002), *Perkinsus olseni* has been reported in Santa Gilla Lagoon (Sardinia) infecting *Ruditapes decussatus*, *Cerastoderma glaucum* and *Venerupis aurea*, in Balearic Islands infecting *Venus verrucosa* and in Delta de l'Ebre (NE Spain) parasitizing *Ruditapes philippinarum*

and *R. decussatus*. (Ramilo, 2015); *P. mediterraneus*, Mediterranean species of *Perkinsus*, has also been described afterwards, infecting the European flat oyster *Ostrea edulis*and numerous other bivalve species along the Mediterranean coasts (Casas, 2004; Ramilo, 2015), *Perkinsus mediterraneus* was detected infecting *Ostrea edulis* from the Gulf of Manfredonia (SE Italy) and Alacant (E Spain), *V. verrucosa* and *Arca noae* from Balearic Islands and *Chlamys varia* from Balearic Islands, Alacant and Delta de l'Ebre (Ramilo, 2015); *P. olseni* and *P. chesapeaki*, have been identified in the Northeastern Atlantic and Mediterranean, in *R. decussatus* and *R. philippinarum. Perkinsus* spp. have been detected in other countries in the Mediterranean basin, namely in Greece, Turkey, Tunisia and Morocco. It has been hypothesized that *Perkinsus* sp. was introduced into the Northeastern Atlantic and Mediterranean Sea through the transfer of *R. philippinarum* from Asia (Cigarría, 1997). This might have been the case of *P. olseni*, since *R. philippinarum* is known to be heavily infected by this parasite in Asia (Park and Choi, 2001), *R. philippinarum* was introduced into France in 1972 by a commercial hatchery (Goulletquer, 1997); it is therefore possible that *P. olseni* was spread in Europe through the introduction of infected *R. philippinarum* from France. To date *Perkinsus* has been reported in more species with studies on its prevalence in the Mediterranean Mussel *Mytilus galloprovincialis* in Japan (Itoh, 2019), in the Balearic Arcipelago (Valencia , 2014) and in Tyrrenian sea (Matozzo, 2018).

3.2 *Pacific Oyster Nocardiosis (PON) of the farmed Crassostrea gigas*

Nocardia is a Gram-positive actinomycete bacteria, it can act as an opportunistic pathogen in terrestrial and aquatic environments (Friedman , 1991). *Nocardia* spp. can infect a wide range of animal taxa, including humans. In molluscs was initially identified in Pacific oysters (*Crassostrea gigas*), the infection resulted in a significant mass mortality in Japan (Takeuchi, 1955), and it was subsequently found in California, Washington, and British Columbia (Elston, 1993). After further characterization the pathogen was described as *Nocardia crassostreae* (Friedman, 1998) and the associated disease renamed PON. Additional research revealed that *N. crassostreae* also affects the flat oyster (*Ostrea edulis*) (Engelsma. 2008; Bower,

2005). An extensive mortality of Pacific oysters *Crassostrea gigas* was reported in Netherlands due to stressful environmental condition and the simultaneous presence of pathogenic *Vibrio* sp. (Engelsma, 2008). In the Mediterranean area, the literature lacks baseline data about distribution of *Nocardia* spp. in molluscs, with very few records in marine mammals and fish (Degollada, 1996; Elkesh, 2012). The first description of *N. crassostreae* infection in the Mediterranean mussel *Mytilus galloprovincialis* was reported in 2013 (Carella et al., 2013) in the Gulf of Naples, due to the introduction in the area through the Pacific oyster *C. gigas* for aquaculture purpose. *N. crassostreae* was reported recently to cause a pulmonary nocardiosis in human, both immunocompetente and immunocompromise patients (Igbaseimokumo, 2016) suggesting the possibility for this pathogen to emerge as an emerging zoonotic disease.

3.3 Multi-pathogen infections associated and Mass Mortality Events (MMEs) in the wild bivalve Pinna nobilis

Mass mortality events (*MMEs*) of the bivalve *Pinna nobilis* were firstly detected along the Spanish Mediterranean coasts reaching up to 100% in early autumn 2016 (Vazquez-Luis, 2017). In 2017–2018, mass mortality events affecting the pen shell *Pinna nobilis* were recorded in two different regions of Italy, such as Campania and Sicily, in the Tyrrhenian Sea (Mediterranean Sea) (Carella et al. , 2019). In this case it was showed that the *MME* were linked to a mycobacterial infection, and the pathogen was identified to be grouped together with *M. sherrisii*, a human mycobacterium, indicating that *Haplosporidium* sp. wasn't the only pathogens involved in the MMEs events in Campania and Sicily (Carella et al., 2019). Mortalities have been reported along all the Mediterranean coasts in the past two years, reaching Greece (Zotou, 2020), Turkey (Ondes, 2020) and at last the Croatian Adriatic coast, considered the last natural shelter against the MME caused by pathogens in pen shell populations (Cizmek, 2020). Since 2018 *P. nobilis* population in Spain has crashed, causing concern and a status change from "Vulnerable" category to "Critically Endangered" with a serious extinction risk

(Orden TEC/1078/2018); and has been included in the IUCN Red List as "Critically Endangered"

4. Emerging microsporidiosis

Microsporidia are obligate and spore-forming intracellular parasites, they include species parasiting aquatic animals and even though are not considered to be foodborne parasites, it has been shown that they can potentially enter the human food chain through water and food.

Microsporidia are characterized by their unique mechanism to infect host cells: The unicellular infectious spores contain the so-called polar filament which coils around the sporoplasm. Upon stimulation, the polar filament is explosively extruded, pierces a new host cell injecting the spore sporoplasm along with its nucleus into the cytoplasm of the host cell. Microsporidia are a very diverse group of organisms with currently more than 1200 species in 143 genera being recognized (Whitner, 1999) and show a very extensive host range infecting nearly all of the invertebrate phyla (especially insects). They are well known as causative agents of economically important diseases in insects (silk worm, honey bees , fish and mammals (rabbits, carnivores).

Before the AIDS pandemic only eight cases of human infections with microsporidia had been reported (Weber, 1994) but in most cases the species identification of the causative agents was not conclusive. Interest in microsporidia increased when they emerged as important opportunistic pathogens during the AIDS pandemic: the first described microsporidiosis in HIV-infected patients with chronic diarrhoea was *Enterocytozoon bieneusi* (Desportes, 1985), followed by *Encephalitozoon hellem* (Didier, 1991) and *Encephalitozoon intestinalis* (Cali, 1993).

The only microsporidian species infecting humans and often occourring in HIV-infected patients with long-standing, well documented animal hosts were *Encephalitozoon cuniculi* which causes infections in rabbits, rodents, carnivores and monkeys (Deplazes, 2000). Microsporidian infections have also been identified in immunocompetent individuals (Curry, 1999) leading to the necessity of major consideration in their role as emerging pathogens.

It is important to understand the route of transmission for emerging microsporidiosis considering the water-food connection that can lead to zoonoses. Many transmissive stages of microsporidia use faeces as major vehicle of transmission, however, the spores of some microsporidia (for example, *Encephalitozoon cuniculi*) contaminate the environment through urine. Contaminated water is an important source of human infection either by direct consumption or by the use of contaminated water in food processing or preparation (Slifko, 2000)

In the summer of 1995, a waterborne outbreak of microsporidiosis occurred in France, with approximately 200 human cases, primarily in the immunocompromised (chronic diarrhoea, dehydration and significant weight loss (Cotte, 1999). Microsporidial spores are stable in the environment and remain infective for days to weeks outside their hosts, their small size (1–5 μm) makes them difficult to remove using conventional water filtration techniques and there is concern that they may possess increased resistance to chlorine disinfection (Shadduck, 1989). Furthermore microsporidiosis may be also a potential meatborne zoonosis since natural hosts of human infective microsporidia are part of the human food chain: for instance *Pleistophora*-like microsporidians may be acquired from raw or partially cooked fish or crustaceans. Some evidence for the foodborne route comes from the incidental finding of microsporidial spores in a human stool sample from an AIDS patient with diarrhoea (MacDougall 1993): spores from the infected fish in this case remained largely intact during passage through the patient's gut, with some of these viable spores initiating the infection.

4.1 Microsporidiosis in Bivalves

In bivalve molluscs, Microsporidia are poorly described, with the only genus reported represented by *Steinhausia* spp. (Sprague, 1972) which infects the oocytes of bivalves and gastropods, and of a another indefinite species that infects the digestive gland of pectinidae and in particular of scallops (*Aequipecten opercularis*). *S. mytilovum* and *S. ovicola* species infect bivalves belonging to the

Mytilidae and Ostreidae families, a Steinhausia-like parasite in *Cerastoderma edule* has also been described.

Steinhausia mytilovum (previously classified as *Haplosporidium mytilovum* and *Chytridiopsis mytilovum*) targets the host's oocytes and in particular is localized both in the cytoplasm and in the nucleus (Sprague, 1972) causing an inflammatory reaction that can affect only small parts of the gonad, surrounding the infected follicle, but in the most severe cases it can infiltrate the gonadal tissue inducing atresia and loss of gonadal architecture. Although this microsporidium critically damages the female gonad, it is not believed to be lethal for the host (Villalba, 1997). *Steinhausia* can be observed in mussels in all reproductive stages, from gametogenesis to spawning (Sunila, 2004).

Even though there are currently some studies reported in areas of the Mediterranean, little is known about its impact, the life cycle in the host and in the environment. Furthermore, totally absent is information in genetic databases that clarify its proximity to other species of Microsporidia.

5. Digenean Trematodes in bivalves

Trematodes are parasites that belongs to the Platelminta phylum, also known flat worms. The trematoda class is divided in two main subclasses: Monogenea, parasites with direct life cycle and Digenea, parasites with indirect life cycle which requires one on more intermediate hosts. The adult Digenea trematodes have a dorsoventral flattened body, a blind-ended digestive system and are equipped with suckers that allow them to attach to the tissues. The biological cycle of these trematodes presents different larval forms (heterogony) which reproduce asexually. This process happens in the intermediate host which, in many cases, as occurs in the most important families, includes both land (gastropod) and aquatic molluscs. The adult parasites are oviparous and lay operculates eggs, the embryo matures inside the egg turning into a ciliate larva known as miracidium. When the miracidium finds the intermediate host it attaches itself to the muscular foot and turns into sporocysts, a sack-like structure containing germ cells. These cells develop in rediae which in the mollusc mutate into cercariae, the last larval stage. Cercariae usually leave the intermediate host and survive in the environment by

encysting, this stage is known as metacercariae and the definitive host, while feeding, breaks the cyst so that the parasite completes its cycle becoming an adult fluke.

Helminths parasiting bivalve molluscs include trematodes, turbellaria, cestodes and nematodes. Trematodes can use the bivalves as main hosts (stages of sporocysts, redie and cercariae) or secondary (metacercaria stage), in other cases the bivalve can represent the main host for all stages of the life cycle. Trematodes can have different target tissues and may cause severe disease in the mollusc leading in a significant economic damage. In bivalves there are many descriptions of trematodes infections in different organs and tissues, in particular gills and gonads, the metacercaria phase is the most described.

Trematodes belonging to the *Fellostomidae* and *Bucefalidae* families can use the bivalve as the main host: the miracidum hatching from the egg infects the bivalve and the larval stages of sporocysts and cercaria develop in the bivalve host as in the case of *Proctoeces maculatus* at the gonadal level, a parasite of mussels. In case of Proctoeces massive infestation, tissue destruction and subsequent castration of the host are observed. The adult stage of the parasite includes vertebrates such as fish and birds. Recently, many mussels have shown the presence of inflammatory lesions at the foot level linked to the presence of trematodes metacercariae or unidentified amorphous structures. To date, there is a single report of trematodes with the same tropism, and in particular, a metacercaria belonging to the group of Echinostomata (Digenea) and to the species Echinostephilla patellae it's reported in the Atlantic mussels' (*M. edulis*) foot in the waters of Great Britain and Ireland.

5.1 Infection by Monorchiidae family in bivalves and fishes from the Mediterranean Sea

The superfamily of the Monorchioidea trematodes includes three families of teleost parasites: Monorchiidae Odhner, 1911, Lissorchiidae Magath, 1917 e Deropristidae Cable & Hunninen, 1942. All the life cycles of the currently known Lissorchiidae and Deropristidae have gastropods as first intermediate hosts, while those of Monorchiidae involve bivalves.The Monorchiidae family consists of over 250 species found mainly in marine bony fishes, with few reports in brackish and

freshwater fishes. Eleven life cycles of monorchiids with identified intermediate hosts are known, all involving bivalves; these monorchiids are phylogenetically diverse, including taxa of eight genera. Another 11 unidentified monorchid cercariae have been reported in bivalves (Wee, 2021).
The life cycle of species belonging to *Monorchiidae* generally require two intermediate hosts. Cercariae are born in sporocysts hosted by marine lamellibranchia and encyst in invertebrates, most often molluscs; the adult forms parasitize the intestines of marine fish. The cercariae belonging to the Monorchiidae have a tail that is sometimes slender, long, with or without expansions of the cuticle, sometimes very short. From the literature the cercariae attributed with certainty to the Monorchiidae are all part of the fauna of North and Central America. As for Europe, some cercariae were placed later in this family. One of the first reports on cercariae parasitizing bivalve molluscs regards *Cercaria longicaudata*, in the Gulf of Marseille (Bartoli, 1966) in the clam *Venus fasciata.*
In Italy, in recent years, monorchids have been described in various specimens of bivalves of commercial interest. A trematode belonging to the genus Postmonorchis (Digenea: Monorchiidae) has been reported to infect mainly gills, but also the labial palps and mantle as well as the intestine, renal epithelium and foot of the wedge clam, *Donax trunculus*, along the Italian Tyrrhenian coast (Campania, Lazio and Tuscany) (Carella et al., 2013).Encapsulated metacercariae of *Lasiotocus longicystis* (Monorchiidae), were described in 2006 in Sardinia, in foot and siphon epithelia of *Ruditapes decussatus* (Culurgioni et al., 2006).
In 2017, the presence of *Postmonorchis sp.* has been reported in autochthonous flat oysters (*Ostrea edulis*) in different locations of the Italian Mediterranean coast (Adriatic, Ionian, Tyrrhenian), however those trematodes were not found in pacific oysters from the same areas (Mancini, 2018).
Complete cycles of monorchids include the cycle of *Paratimonia gobii* (Trematoda-Monorchiidae), (Prevot & Bartoli, 1966) parasite of the goby *Pomatoschistus microps* (Teleostea gobiidae), with its first intermediate host a bivalve, *Abra ovata* (Lamellibranchiata, Scrobiculariidae). *Gymnocephalous* cercaria leaves the mollusc through the siphons, and then encyst in the syphon of another Abra intermediate host. The accumulation of metacercaria results in the necrosis and

detachment of the infected siphon which, by becoming part of the food chain of the final host, allows the continuation of the cycle (Maillard, 1975). Another complete cycle, described by Bartoli (2000) is the life cycle of *Cercaria cerastodermae I*, a parasite of *Cerastoderma edule,* recorded for the first time in the Atlantic Ocean off the Iberian Peninsula. Most of the cercariae in were detected within sporocysts and were described to be encysted as metacercariae in the hemolymph of the digestive gland, in the gonads, in the gills and in the foot of the mollusc. The adults were experimentally obtained by infecting specimens of *Diplodus sargus* and compared with adults of *Monorchis parvus* collected from *Diplodus annularis* along the French Mediterranean coast. The comparison between wild and experimental adults allowed to identify the adult stage of *Cercaria cerastodermae I* as *M. parvus*. Other descriptions of *Monorchis parvus* occur in the populations of *Cerastoderma edule* of the North Sea in Germany (Thieltges, 2006)

Other Monorchidae species found in mediterranean fishes are:

- *Monorchis monorchis*, parasite of *Spondyliosoma cantharus*, with *Antedon mediterranea* (Echinodermata) as its intermediate host. (Prevot, 1967)
- *Monorchis hermani*, in *Sparus aurata* (Issa, 1963)
- *Monorchis blennii* sp. in *Parablennius gattorugine* (Jousson , 2002)
- *Ancylocoelium typicum*, in *Trachurus trachurus* (Bartoli, 2004)

For some of those the intermediate host has not been reported

6. Emerging Diseases in the Mediterranean mussel Mytilus galloprovincialis in the Thyrrenian Sea: previous reports, studies and premises

The Mediterranean mussel *Mytilus galloprovincialis* production has an important tradition in Italy, making mussel are as source of income from aquaculture. Italy covers over 70 percent of the production of Mediterranean mussel and is the Shellfish companies account for over 50 percent of the total number of farms and contribute to 63 percent of total aquaculture production (FAO, 2015). Mussels are

mainly farmed in Emilia Romagna, Veneto and Apulia, but also in Marche, Sardinia and Campania. Mediterranean mussel is quantitatively the most important species. In 2013, 64 235 tonnes were produced, which represent about 72 percent of shellfish production. (FAO, 2020) Productions significantly fluctuated in the past decade, mainly due to bureaucratic issues, environmental problems (algal blooms) and extreme weather events taking at date the production at 50.338 t in 2020 (MIPAAF, 2020). The official average mussel productivity in Campania Region is around 15.000 quintals which are, for the major part, absorbed by the local market, whereas other sources estimate the production around 30,000 quintals. One of the issues with regional mussel production is represented by the large quantity of mussels is introduced into the market illegally, without sanitary control (Anastasio et al., 2004). In Campania there are about 12 farming centers and the species of molluscs commonly bred are the Mediterranean mussel (*Mytilus galloprovincialis*) and different clams including the native grooved carpet shell (*Tapes decussatus*) and the manila clam (*Tapes philippinarum*).

Mussels, as all the filter feeders, are exposed to a variety of natural stressors that, along with genetic differences in susceptibility to stress, result in an increased variability of molecular and tissue responses (Marigomez, 2013). Mussel are often used as sentinel organism in environmental surveillance projects for their ability to being resistant to pollution. Research showed the ability of mussels to concentrate and to retain bacteria into their tissues (De Donno, 2008) and to concentrate heavy metals such as lead and biotoxins in their lysosomal system (Regoli and Orlando, 1993).

Expression of disease in mussel can be considered as the failure of an adaptative effort of the organism to a prolonged stress condition due to different physical, chemical, biological factors, the response can result in a successful adaptation or in worst cases death of the animal (De Vico and Carella, 2012).

Mass mortality episodes, which can affect both juveniles and adults, are a significant problem for the aquaculture sector and can result in significant financial losses. Beyond the practical implications, the study of disease is becoming crucial for maintaining animal biodiversity because it can alter the interactions between hosts and species as well as those between coexisting species and their predators

and prey, which in turn affects an ecosystem's structure and equilibrium. The presence of parasites and diseases in mussels has been widely documented and may cause severe damage and contribute to decreases in both natural and cultivated populations (Carella et al., 2015). Although the continuous changing in environment, the appearance of novel pathogens and the increase of the incidence of known diseases indicates that further studies are necessaries for emerging and unidentified pathogens in mussels.

Historical data have shown the constant presence of undescribed pathogens belonging to the group of microsporidia and trematode in *M. galloprovincialis* in Campania Region, although scarce are the information in literature about their emergence and effect on the host.

The present study focused on two pathogenic species observed in mussels along the Campania coastline, in order to characterize their occurrence, the associated lesions, the effect on the host and define a molecular classification:

- *Steinhausia mytilovum,* an ovarian microsporidian parasite of *M. galloprovincialis*;
- Metacercariae of an unidentified digenean trematode in the foot of *M. galloprovincialis*.

CHAPTER 2

THE MICROSPORIDIUM *STEINHAUSIA MYTILOVUM* (FIELD, 1924) AFFECTING THE MEDITERRANEAN MUSSEL (*M. GALLOPROVINCIALIS*) ALONG THE COASTLINE OF CAMPANIA REGION (ITALY): DISEASE, EPIDEMIOLOGY AND MOLECULAR DIAGNOSTIC

1. Introduction

Microsporidia are a spore-forming, obligate, intracellular parasites infecting all major taxa in all environments (Becnel, 2014). Of the 187 genera described, almost half are known to infect aquatic organisms, mainly teleost and crustaceans. Although not currently considered to be foodborne parasites, it has been shown that microsporidia can potentially enter the human food chain through water and food.
In bivalve molluscs, Microsporidia are rarely described, with the only reported genus represented by *Steinhausia* spp. (Sprague, 1972) which infects the oocytes of bivalves and the digestive gland of the scallops (*Aequipecten opercularis*) (Lohrmann, 1999). *S. mytilovum* and *S. ovicola* are reported to infect bivalves belonging to Mytilidae and Ostreidae families, another *Steinhausia*-like parasite has also been described in *Cerastoderma edule* (Carballal , 2001).
Steinhausia mytilovum (previously classified as *Haplosporidium mytilovum* and *Chytridiopsis mytilovum*) targets the host's oocytes and in particular it can be found both in the cytoplasm and in the nucleus (Sprague, 1972) causing an inflammatory reaction that can affect only small parts of the gonad, surrounding the infected follicle in the most severe cases it can infiltrate the gonadal tissue inducing atresia and loss of architecture of the gonad. Even though this microsporidium can critically damage the female gonad, it is not considered to be lethal for the host (Villalba, 1997). *Steinhausia* can be observed in mussels in all reproductive stages, from gametogenesis to spawning (Sunila, 2004).

Currently scarce are the studies about the distribution and the effect of the potential impact of *Steinhausia* in the Mediterranean bivalves. For decades, interest in microsporidia remained centered on their cellular organization and taxonomy other than pathogenesis in humans and animals of economic importance, but the overall interest on microsporidia was shadowed by more apparently important bivalve pathogens.

Phylogeny of microsporidia is currently based on analysis of SSU rRNA genes. They are closely related to fungi, although their taxonomic status (as a clade within the fungi or a sister clade to the fungi) remains the subject of debated. They possess the smallest genomes of any eukaryotic organisms, making them highly dependent on the host. Currently there is no information about *Steinhausia* in genebank. SSU rRNA gene is the most common target for molecular confirmation of microsporidian infections and has been extensively applied for taxonomic purpose to supplement data gathered by classical methods, based on spore morphology, developmental sequences and life cycles characteristics.

The aim of the study was to report the presence of *S. mytilovum* in mussels (*M. galloprovincialis*) over the years both in natural beds and in farms along the coast of the Campania Region, in order to describe the lesions caused by the parasite and to carry out for first time a molecular classification of the genus.

2. Materials and Methods

2.1 Animal sampling and sample preparation

The present study was carried out on the Mediterranean mussel *M. galloprovincialis* in 13 farms and 1 natural bed starting from 2009 to date (using the archive from the Laboratory of Pathology of Marine Organisms for the retrospective study), with a total of 469 examined animals. Animal sampling was performed in different areas of the coastline of Campania region: Bacoli, Bagnoli, Napoli Castel dell'Ovo, Castellamare di Stabia, Lucrino, Miseno, Nisida, Torre del Greco. (Figure 1)

Collected samples were transported alive and kept in insulated coolers at +4 °C to the laboratory. In order to limit any animal stress and subsequent analysis artifact that may result from the sampling and transport process. The animals were submitted to macroscopic examination of the valves and meat and observed under a stereomicroscope in order to evaluate any external lesions. Biometrical parameters were measured for each animal: total weight (TW), meat weight (MW), shell weight (SW) and length were measured for each animal.

The mussel tissues were preserved for macroscopic and microscopic examination (optical and electron microscopy - SEM / TEM) and for molecular diagnostics.

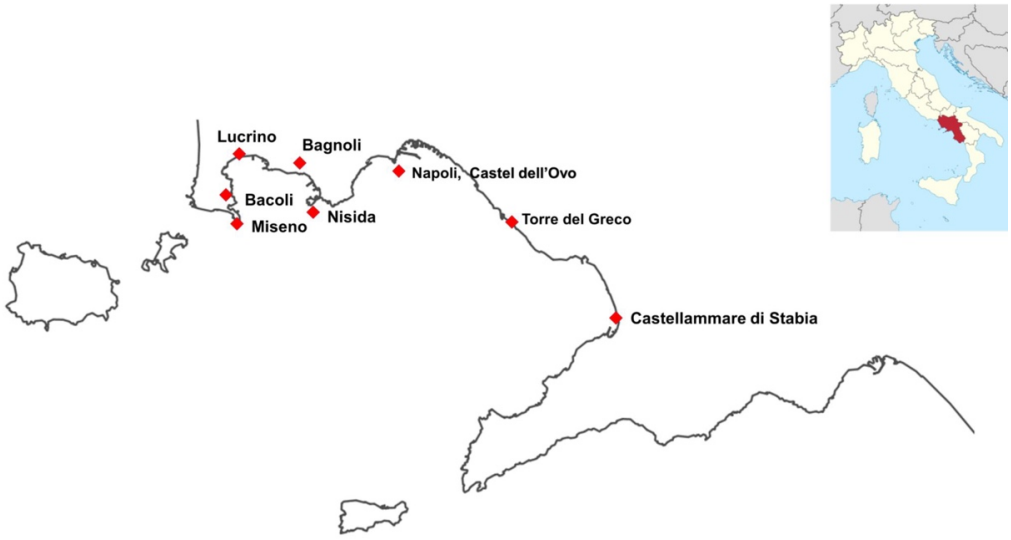


Figure 1: Map of the coast of Campania (Italy) with the location's sites.

2.2 Animal histopathology

For microscopy, from each animal a transverse section comprehending digestive gland, gonad, gills and mantle was taken and put into histological cassettes with sampling code and animal number. The cassettes were placed in Davidson's solution with acetic acid for 24 hours at room temperature. The day after they were moved to Davidson's solution without acetic acid and left for 10 days at room

temperature. After the fixation process samples were dehydrated and embedded in paraffin by using a caroselle histokinette. The final embedding was performed manually to include the samples in paraffine blocks, the samples were cut at the microtome to obtain sections of 3-4 μm thick. Moreover, for molecular diagnostics, small pieces of digestive gland, gonads and gills were taken and placed in TRIS-EDTA and stored at -20°C.

In order to define *Steinhausia* phases of development and mussel response, we performed different stainings: Haematoxylin-Eosin as normal routine staining, and additional special staining like Trichrome Masson, VOF, Ziehl-Neelsen, Feulgen, Gram.

The inflammatory response was classified at the microscope according to the observed morphology, the degree of infection and the damage associated with atresia. A grading from 0 to 3 was attributed to the inflammatory response using a scale based on the disease intensity: 0 = no inflammatory response; 1 = infiltrative response; 2 = encapsulation; 3 = strong inflammation associated with atresia.

For each animal six developmental stages were distinguished by the microscope in mussel gonads: stage 0 (resting stage); stage I (early development); stage II (progression of gametogenesis); stage III (ripe), stage IV (spawned gonad); stage V (reabsorbing stage), Seed (1975).

Infection intensity (%) was also evaluated at optical microscope by counting infected oocytes containing visible sporogony in cytoplasm or nuclei.

2.3 Statistical analysis

Biometrical parameters were used to evaluate the Condition Index (CI) of all animals as reported by Lucas and Beninger, 1985.

$$CI = \text{meat weight/total weight -shell weight} \times 100$$

The prevalence and infection intensity were examined in each sampling. Animals were sorted in two categories, diseased and healthy. Data were normally distributed for all areas. Descriptive statistics with mean values for SW, TW, MW and CI were evaluated for the mentioned groups. The Spearman Correlation was

used to assess statistical evidence for a linear relationship among the same pairs of variables in the population. The level of statistical significance considered was p <0.05. Statistical analysis was conducted using SPSS statistics v. 27.0

2.4 Amplification and cloning of SSU rDNA

The samples that tested positive on microscopic examination with an high intensity of infection were processed for molecular diagnostics. DNA was extracted from infected gonads stored in TRIS-EDTA at -20° using the DNAeasy Blood & Tissue Kit (Qiagen) that provides a silica based DNA extraction in spin-columns.
Different primers set were tested to amplify the small subunit ribosomal RNA gene (SSU rDNA) from Vossbrick et al., 1987 and Baker et al 1995, McClymont et al., 2005. After different attempts (Table 1) the used primers 530F/1342R.

Primer Name	**Primer sequence 5' - 3'**
CTF/V1	CACCAGGTTGATTCTGCCTGAC
530F	GTGCCAGCMGCCGCGG
1342 R	ACGGGCGGTGTGTACAAAGAACAG
18sF	CACCAGGTTGATTCTGCC
580 R	GGTCCGTGTGTTTCAGACGG
964 R	CGCGTTGAGTCAAATTAAGCCGCACA
350 F	CCAAGGAYGGCAGCAGGCGCGAA

Table 1: primers set from SSU rDNA for microsporidians. In light green the primer pair that allowed us to obtain the final amplicon (530F/1342R).

PCR were performed, containing 10 µl of 5X PCR buffer, 1 µl of each dNTP (20mM), 0,5 µl P*latinum SuperFi Taq Polymerase,* 10 pmol of each primer (530F/1342R), 2 µl of DNA template (50ng/ul), and deionized water in a 50 µl reaction volume. Thermocycler settings for 530F/1342Rwere 94°C for 4 min,

followed by 35 cycles of 94°C for 1 min, 60°C for 40 s, and 72°C for 1 min and then 72° C for 7 min. Amplicons with the correct size were subsequently purified through (1300 bp) were excised from the gel and purified by using DNA/RNA and protein purification kit (Macherey- Nagel). A second PCR was performed on the purified fragment with same primers and conditions resulting in a 900bp amplicon. The amplicon was cloned into a *Pgem Teasy vector* (Promega) as described by the manufacturer. and send to sequencing service (*Eurofin Genomics*).

2.5 Phylogeny

The nucleotide sequences SSU from different Microsporidia species were downloaded from GenBank and aligned with the corresponding region of the sequence obtained in this study. The sequences obtained of SSU rDNA were aligned using the MEGA X software (Kumar. 2018). Neighbor Joining Method (NJM) trees were then constructed.

2.6 Electron Microscopy

Three samples of oocytes infected by *S. mytilovum* from different areas were processed from 2.5% glutaraldehyde, post-fixed in 2% OsO4, and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate. Ultra-thin sections were collected on carbon coated Formvar 200 mesh grid post-stained with 2% aqueous uranyl acetate. Sections were examined with FEI Tecnai G2 S-twin transmission electron microscope (University of Naples Federico II, Italy) operating at 110 kV (LaB6 source).

3. Results

3.1. Animal histopathology

Steinhausia life cycle, described by Sprague (1972) and after by Sagristà (1998), includes different stages of development, the spore releases the polar tube in the cytoplasm of the host cell, initiating the infection and starting the merogonial proliferative phase, in the successive sporogony phase immature spores are firstly formed, spores become infecting and mature when the formation of the polar filament and anchoring disk is complete. (Sprague, 1972; Sagristà 1998).

Upon light microscopy, with routine stains H&E, the meront phase described by Field (1964), represented by uni nucleated cells, was detected only in a few samples (Figure 2A). *Steinhausia* presence was primarily detected in its mature form of sporophorous vesicle of ~ 10 µm, delineated by a membrane and containing a variable number of spores (in a number of one, three per cell) of 3-5µm, located mostly in the cytoplasm, or nucleus of the oocyte. Selective stains were used to detect *S. mytilovum* phases of development, mature spores appeared positive for Ziehl-Neelsen stains appearing purple to pink, whilst immature spores were not stained, remaining blue (Figure 2E). At trichrome V.O.F stain (Light Green-Orange G-Acid Fuchsin) the spores appeared stained from light pink to intense orange, depending on the phase of development (Figure 2F).

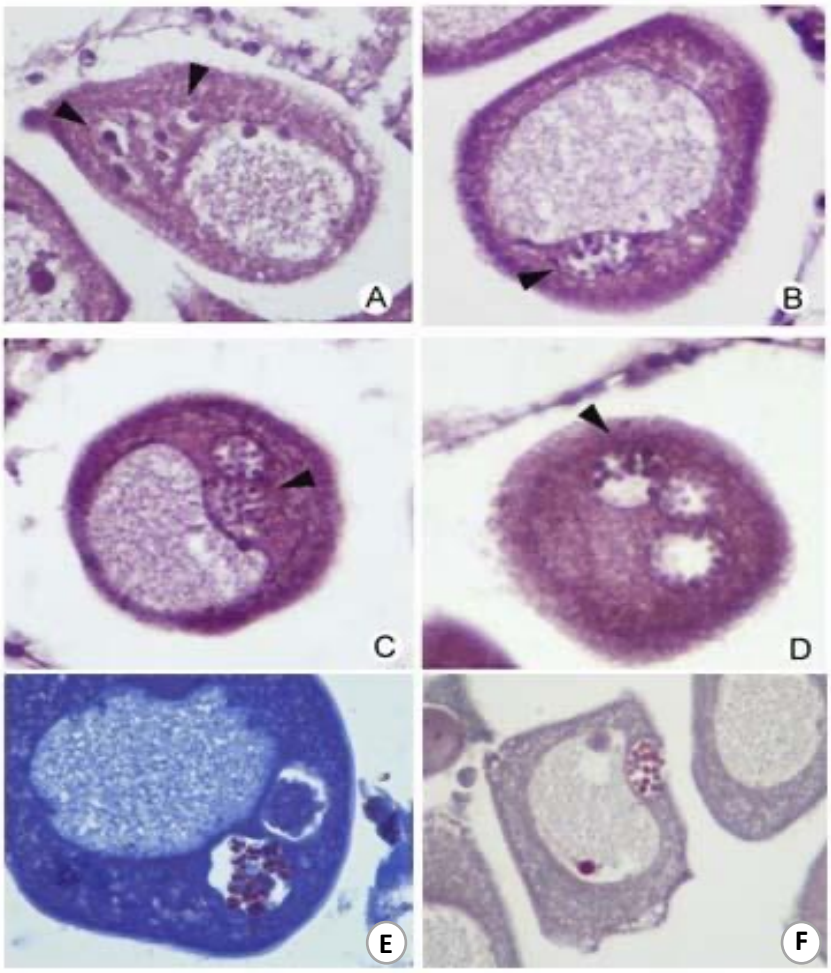


Figure 2: *Steinhausia* presence was detected in the form of meronts (A) but primary as cysts within a sporophorous vesicle delineated by a membrane and containing a variable number of spores, in a number of one, three per cell, at cytoplasm and at nuclear level (B-D). Mature spores appeared positive for Ziehl-Neelsen (E), and V.O.F. stain (F).

Lesions were classified according to the type of inflammatory response observed (as previously described in materials and methods). Infected mussels showed a variable inflammatory response represented in the first early stage by inflammatory infiltrates within gonadal follicles and vesicular connective tissue (Figure 3A) followed by encapsulation with hemocytes surrounding infected follicles (Fig 3B); in most severe cases a severe loss of gonadal tissue could be observed with fibrotic follicles and atretic oocytes. (Figure 3)

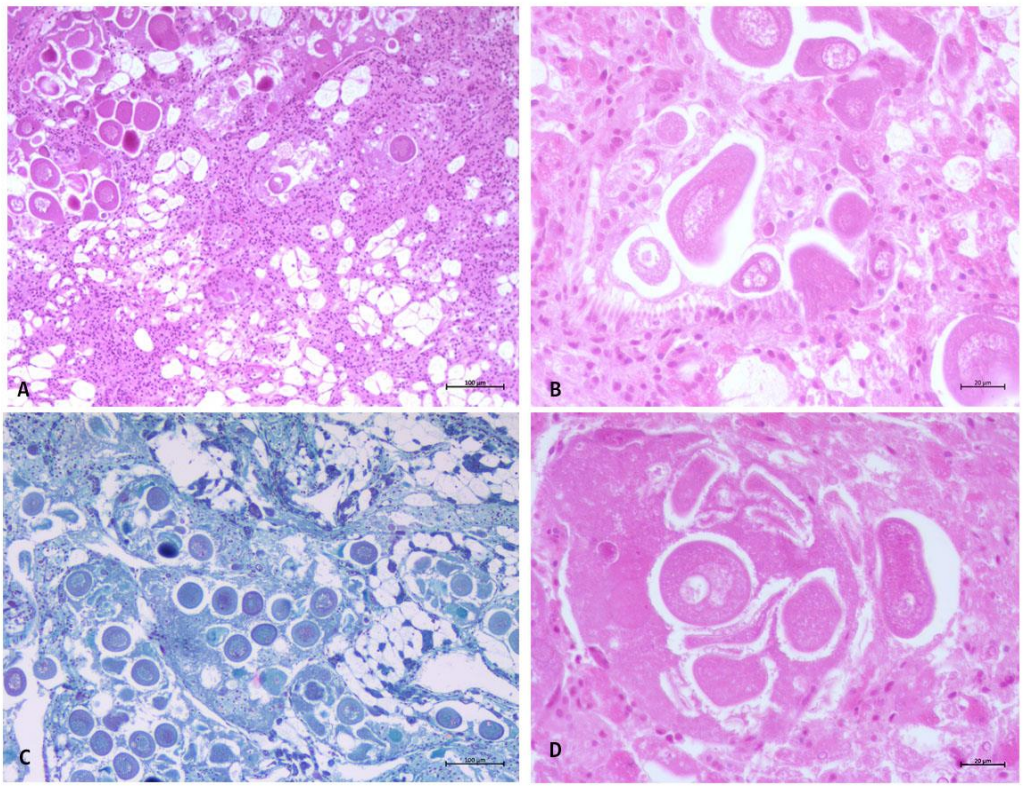


Figure 3 *S. mytilovum* lesions: lesions were classified according to the type of lesions observed A) strong infiltrative inflammation in the gonad with tendency to nodulation (B) (H&E); C) inflammatory lesions accompanied by gonadal atresia (V.O.F Stain) and detail of follicle damage (D) (H&E).

3.2 Disease distribution and effect

Descriptive statistic of CI (Condition Index), Size, MW (Meat weight) and TW (Total weight) of the mussels showed different ranges of weight and dimension along the sampling areas and the seasons. The largest number of samples was collected in Miseno (161) with a wide size range (1,8 cm the smallest-7,3 cm the biggest) and total weight between 2,85 g and 80,88 g. The presence of *Steinhausia* was detected in 9 out of 13 farms with a variable prevalence, from 2 to 30%, depending on the area and the season. The female/male specimens' ratio was 45% female, 44% male with an 11% animals of undetermined sex due to gonadal resting stage. Amongst the female population 90 animals did not present any sign of gonad

infection, whilst 123 resulted infected with *Steinhausia*; diseased animals were found in all the months in which sampling was conducted (Figure 4)

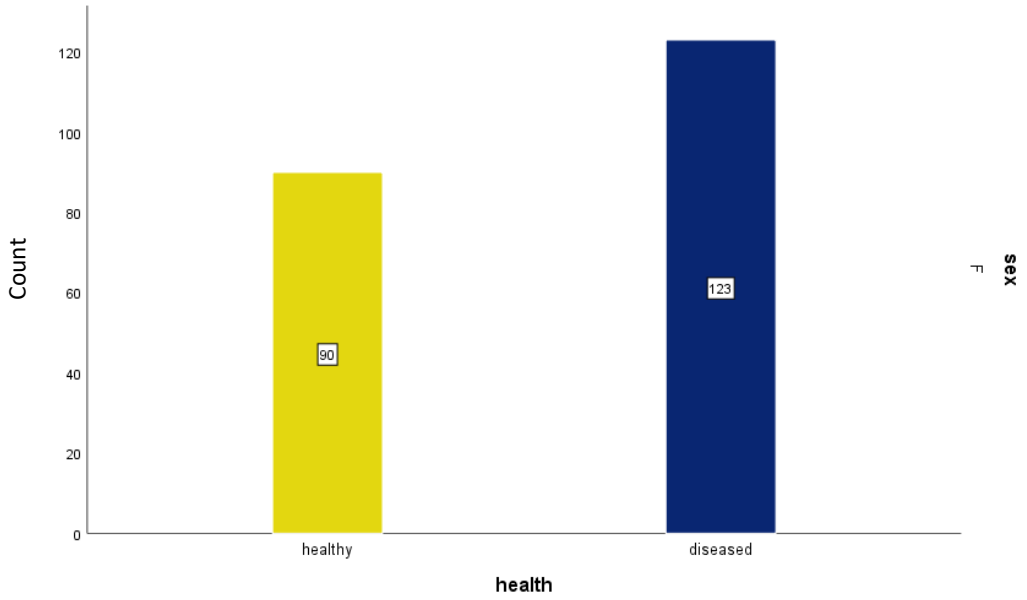


Figure 4: *M. galloprovincialis* female specimens affected from mussel egg disease (123) during the period of sampling (2009-2021)

Most of the infected subjects were found in the months of March and May in specimens with an advanced stage of gonad development (ripe- spawn).

In Capo Miseno area, from 2009 to 2020, the parasite has been reported in all the samplings, in every season with an overall prevalence of 37%. (Figure 5) In some the atretic phenomena of the oocytes prevailed over the inflammatory ones. *S. mytilovum* elicited an infiltrative (24,8%) or a strong capsular inflammatory response (43,4%) in gonadal follicles and surrounding vesicular connective tissue, in some case accompanied by gonadal atresia (24,8%), eventually associated to loss of gonadal architecture. In other cases (7%) no reaction was observed (Figure 6).

The statistical analysis of mean condition index showed statistically significant differences between infected and heathy animals: mean values of MW, SW, TW showed lower values for the sick animals and for all the parameters under

examination with a 95% statistically significant difference (4,13 g D / 4,41 g H for MW; 6,19 g D / 6,54 g H for SW; 16,00 g D / 16,27 g H for TW). (Figure 7).

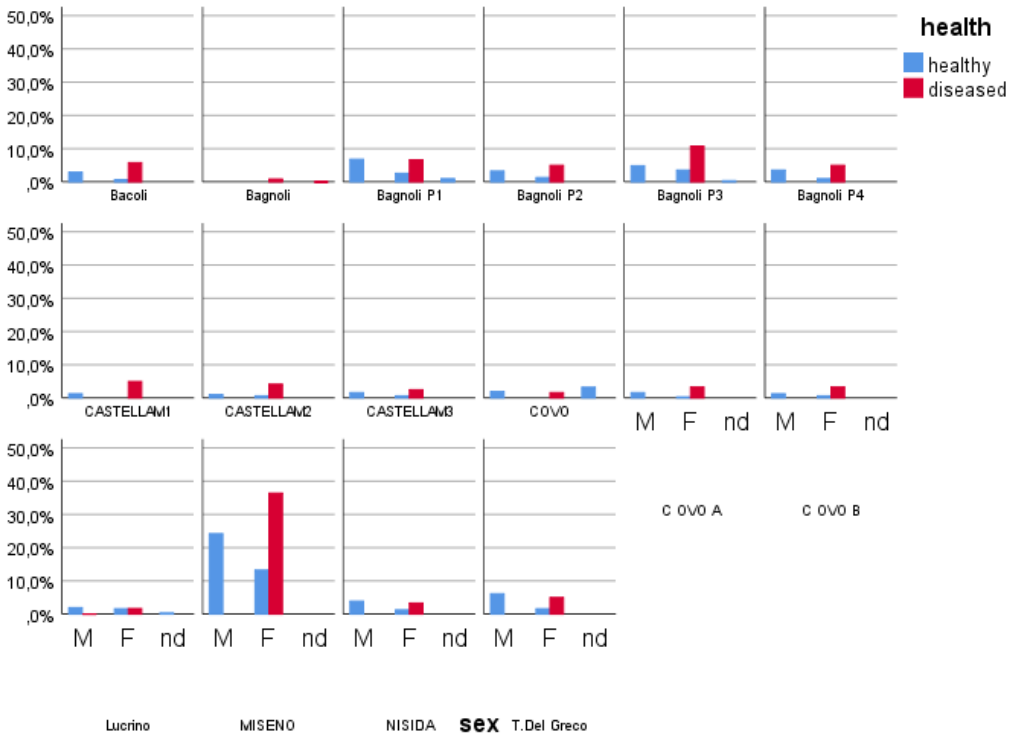


Figure 5: Prevalece of *S. mytilovum* in all the sampled locations, all areas revealed the presence of the parasite with the highest prevalence in Miseno (37%).

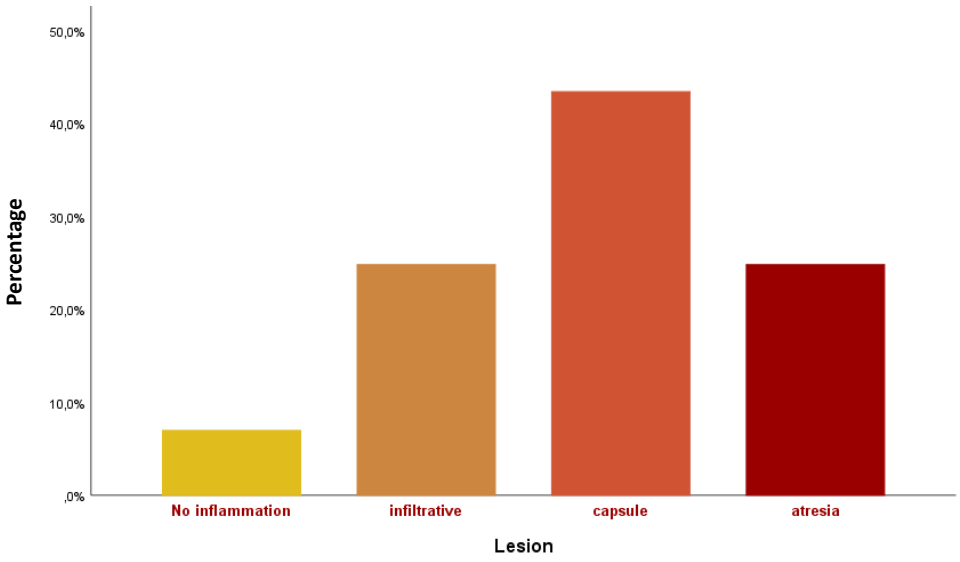


Figure 6: Prevalence of different types of lesions

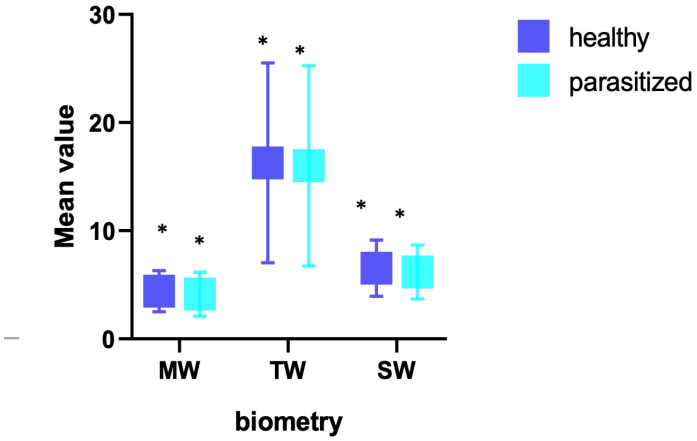


Figure 7: Comparison of the biometry related to the pathogen between diseased and healthy mussels for the value Meat Weight (MW), Total Weight (TW) and Shell Weight (SW). * statistically significant p= 0.05

3.3 Molecular diagnostics

Samples resulted histologically positive for *Steinhausia* resulted in an amplicon of 900bp at the PCR.

Blast results showed an identity of 83% with microsporidia sequences not yet classified. Microsporidian systematics currently turn around five taxonomic clades, identified by *SSU* ribosomal gene sequence data. *Neighbor Joining* of the 18S assigned *S. mytilovum* in a separated branch within the Clade V, defined as the Class Terresporidia, with closest genetic relationship (84% identity) to an undermined invertebrate's ovarian microsporidian (Figure 8).

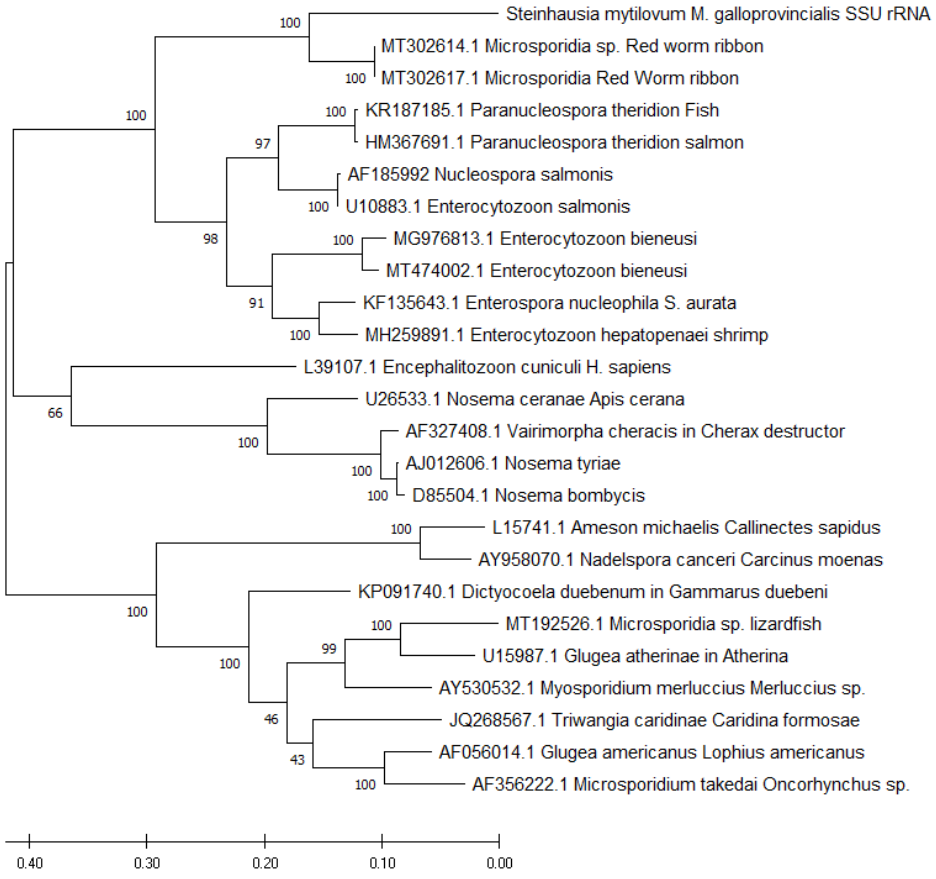


Figure 8: NJM tree based on SSU rRNA gene sequences for *S. mytilovum*

3.4 Electron microscopy

Semithin sections of oocytes infested by *S. mytilovum* showed vacuoles that contained different developmental phases of *Steinhausia* in the form of sporonts, sporoblasts and prespores.

The developing spores at sporoblast stage were the most represented in the vacuoles and appeared round in shape and displayed visible organelles, with an anchoring disk connected to a polar filament and a developing laminar polaroplast; the nucleus in the pre-spore stage appeared with a dyplokariotic aspect. In most cases, during the development of the parasite, at the periphery of the sporophorous vescicle we observed the presence of the host mitochondria, sometimes with signs of idropic degeneration and cristolysis. Mitochondria were also found in the parasitic vesicle, in the number of two or three. (Figure 9)

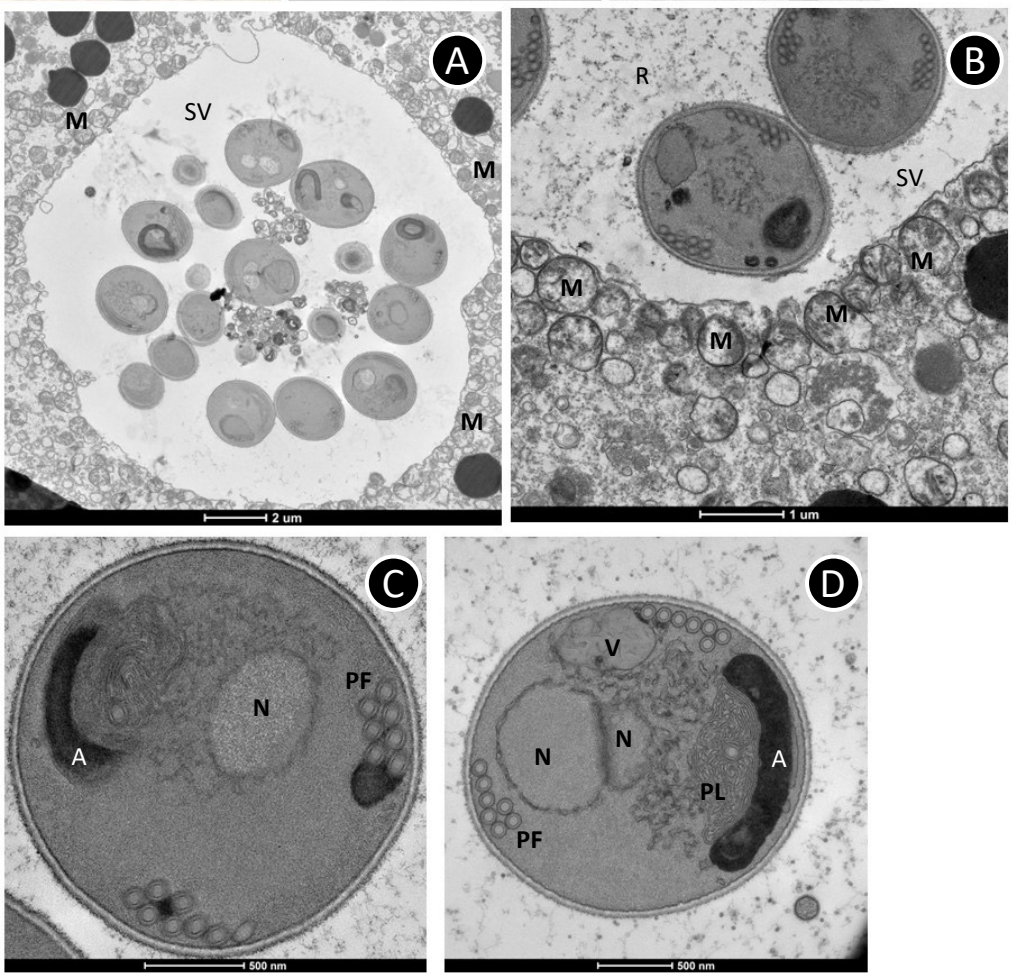


Figure 9. Ultrastructure of early development of *S. mytilovum* in mussel's oocyte: **A.** Sporophorous vescicle (SV) filled with forming spores and peripheral mitochondria **B.** Grouping mitochondria (M) around the SV and free ribosomes (R); **C.** early sporoblast displaying one nucleus (N) and sparse genetic material, small anchoring disk (A) surrounded by and a polar filament (PF) **D.** Late stage sporoblast with similar features to **C** but with a more developed anchoring disc, showing dyplokaria, lamellar polaroplast (PL) and a lateral vacuole (V).

4. Discussion

Microsporidia are a group of intracellular and unicellular parasite, infecting host from all taxa in all environments, half of the known microsporidia infect aquatic organisms, from fish to invertebrates, protists and hyperparasites of aquatic hosts. (Stentiford, 2013). *Steinhausia mytilovum* is a microsporidian parasite that infects mussel oocytes with a wide distribution, *S. mytilovum* has been reported in *Mytilus edulis* in the Atlantic and Pacific oceans and in *M. galloprovincialis* in European waters. In this study we provide an insight on the microsporidian pathogen *Steinhausia mytilovum* with a long-term study on the parasite pathogenicity and damage to the host and bringing a fist molecular diagnostic classification.

4.1Histopatology and effect on the host

Animal histopathology showed that *Steinhausia* can be responsible of various type of lesions on the mussel's gonad such as haemocyte infiltration and capsules , leading in the most severe cases in loss of gonadal tissue, and oocyte atresia as reported in other invertebrates (Pan, 2018).

Routine and special stains were used to observe *Steinhausia*, with the Hematoxylin and eosin allowing us to observe more easily and with low magnification areas of encapsulation in follicles in which the microsporidia were detected mostly in its mature form of spores and pre-spores. Special stains enhanced the detection and visualization of the pathogen and VOF stain turned out to be more efficient in detection and count of infected oocytes.

In our study we were able to demonstrate that high infection intensity negatively impacts on the host, due to a less developed gonad resulting in lower values for MW and CI.

In line with Villalba et al. (1997) and Bigas et al. (2000), this parasite negatively impacted the CI of infected mussels in this investigation and induced a robust hemocyte infiltration response. A correlation between *S. mytilovum* infection and the mussel reproductive cycle, which intensified throughout the spawning season, was also noticed in this study.

The highest prevalence was found in Capo Miseno area (37%) and higher than the one found in the females of *Mytilus galloprovincialis* in previous studies in the Gulf of Naples (10%) (De Vincentis and Renzoni, 1963), or in cultured mussels as in Lake Fusaro (15%) (Ceschia, 2003). Other European areas reported lower prevalence, 35% in North Egean Sea (Rayyan & Chintiroglou, 2004) and Galicia (28,3%) (Villalba, 1997). The highest value in the Mediterranean though was reported by Sagristà et al. (1998) with a 50% prevalence. The presence of this parasite has been recurrent in the past years and its impact has resulted in loss of gonadal tissues and subsequent lower weight of the mussels, meaning, in consequence of a potential large spread of the parasite in a mussel farm, money loss for farmers.

4.2 Ultrasructure of Steinhausia mytilovum and pathogenetic features

Steinhausia mytilovum was characterized at ultrastructural level, allowing us to describe the development stages of this microsporidium. The first ultrastructural description of *S. mytilovum* was reported by Sagristà et al., 1998 defining the different phases of the pathogen development.

Steinhausia, according to Sagristà et al. (1998) possesses a polar filament, which allows the parasite to invade the host cells and transfer microsporidia sporoplasm; polar filament had a circular transverse section and not a honeycomb surface as reported for *S. brachynema* (Richard and Sheffield 1970) and associated as a common characteristic to all the species of the genus *Steinhausia*. Polar filament first forms in the sporonts close to the electron lucent central vacuole and reaches

its complete development in pre-spores, where it connects to a membranous tubular network structure and an electron dense posterior vacuole. In our study we observed the merogonial plasmodium and the sporonts exhibiting diplokaryon and the formation of the sporophorous vesicle surrounded by the host cell mitochondria. Microsporidia development depends strictly on the host, they do not have mitochondria, whilst presenting similar structures called mitosomes which do not produce ATP (Williams, 2002)(Tsaousis, 2008).

In this study, the strict contact of the parasitic vacuoles with the mitochondria of the host cell highlights how the microsporidia, which do not have this type of organelle, rely on the host's energy supplies for their survival, as described also in the human microsporidian infection by *Encaphalitozoon* (Han, 2019). In our study transmission electron microscopy (TEM) showed the presence of mitochondria also inside the parasitic vesicle, this facilitates the sporonts growth. In our study this host-pathogen interaction is described for the first time. Moreover, mitochondria associated with parasitic vescicles showed various signs of damage, from hydropic degeneration, to lysis of mitochondrial membrane and apoptosis, all these alterations to the energetic system could be responsable to oocytes degeneration.

4.3 Phylogenetic analysis

Microsporidian phylogenies was determined by previous studies by using the small-subunit (SSU) rRNAgene. Three environmentally defined groups (Aquasporidia, Marinosporidia, and Terresporidia) were classified, those groups were initially divided into five genetically distinct clades, denoted by Roman numerals (I, II, III, IV, V)(Vossbrink, 2014). Our results showed similarity of *Steinhausia mytilovum* (84%) to unclassified ovarian microsporidia, the closest described species is from a Nemertean, *Maculaura alaskensis*, at oocyte level. In this study we provided the first molecular description of *S. mytilovum*, assigning this microsporidium, with the results of the Neighbor Joining of the 18S, in a separated branch within the Clade V.

Clade V is composed mostly of microsporidia that have been isolated from aquatic animals, such as marine fish and crustaceans, fish with life cycles that include both

freshwater and marine habitats, fish and crustaceans that live in freshwater ecosystems, and one marine nemertean host (Ardila-Garcia & Fast 2012). However, no terrestrial microsporidia were assigned to clade V. First taxonomic morphological description by Field report *S.* as *Haplosporidium mytilovum* (Field, 1924). After that, Sprague (1972) transferred it to *Chytridiopsis* and later the same author (1992) created the microsporidian family *Chytridiopsiade* and the genus *Steihausia*, with three species, *S. ovicola*, *S. mytilovum* and S. *brachynema*. Then, Larsson 1988 defined the genus *Steinhausia* as a microsporidian pathogen develops in association the nuclei of the cell host similarly to the other species *S. brachynema*. Actual classification on WORMS (World register of Marine Species) report it in the subclass *Dihaplophasea*, order *Dissociodihaplophasida* where families of *Nosematidae* and *Pseudopleistophoridae* are placed.

Steinhausia mytilovum share less than 90% sequence similarity to all currently described microsporidian species. At the nucleotide level, molecular analysis may complement the morphological diagnosis and aid in species identification of microsporidia.

CHAPTER 3:

FIRST REPORT OF A DIGENEAN PARASITE IN THE FOOT OF THE MEDITERRANEAN MUSSEL (*M. GALLOPROVINCIALIS*) IN CAMPANIA REGION (ITALY)

1. Introduction

Bacterial and parasitic infections are commonly reported in bivalve molluscs belonging to the genus *Mytilus* sp., in particular at the level of the digestive gland, gonads and gills, but are rarely described at the level of the foot. Microscopic assays conducted from 2008 to today at the laboratory of Pathology of Aquatic Animals of the Department of Biology of the University of Naples Federico II have identified the systematic presence of metacercariae belonging to the group of helminth trematodes at the foot level of the examined mussels.
Trematodes can have a variety of target tissues, which can result in serious damage in the mollusc and major economic loss. The metacercaria phase of trematodes infections in bivalves is the most well-documented and affects several organs and tissues, particularly the gonads and gills. Recently, many mussels have shown the presence of inflammatory lesions at the foot level linked to the presence of trematodes metacercariae or unidentified amorphous structures.
To date, there is a single report of trematodes with the same tropism, in particular in Atlantic mussels (*M. edulis*) in the waters of Great Britain and Ireland and reported to belong to the group of Echinostomates digenea, and to the species *Echinostephilla patellae*. (Prinz, 2009)

The purpose of the following study was the identification of the metacercariae infesting the foot of the examined mussels, with a description and characterization of the macroscopic and microscopic lesions other than perform a first molecular identification of the trematode species.

2. Materials and methods

The study was conducted on *M. galloprovincialis* in 13 mussel farms and natural beds along the coasts of the Campania Region, with a total of 323 animals from 2008 to today in the areas of: Bacoli, Bagnoli, Castellamare di Stabia, Miseno, Nisida, Torre Annunziata, Villaggio Coppola (Caserta).
At the time of sampling, the mussels were collected for macroscopic, microscopic and molecular diagnostics. During current sampling, 165 specimens were observed under a stereomicroscope in order to evaluate any external lesions. Moreover, the foot was dissected in correspondence with the ventral groove from which the byssus originates and observed in order to evaluate the presence of lesions or parasites in cut section.

2.1 Animal microscopy

Animal's foot and visceral mass were processed for light microscopy and embedded in paraffin. Routine and special staining were performed to evaluate anatomy of the foot e, parasite prevalence, and lesions related to the parasite: Hematoxylin and Eosin, Masson's Trichrome, PAS-BA. The lesions were classified under the light microscope and divided into visible cercariae, inflammatory nodules with no visible pathogen and fully visible metacercariae.

2.2 Molecular diagnostic

Light microscopy positive specimens exhibiting a high infection rate, with multiple metacercariae present in the foot were processed for molecular diagnostics. DNA was extracted from paraffin with DNAeasy FFPE kit (Qiagen). After using different primers (Table 2) the final PCR was performed using primers to amplify the barcode region of the mitochondrial nuclear 18S rRNA gene of Platelmints, the pair of Primers used were: CFor and ARev (Routtu, 2014).

Primer Name	Primer sequence 5' - 3'
COI F Trema	5' TTTTTTGGGCATCCTGAGGTTTAT 3'
COI R Trema	5' TTTTTTGGGCATCCTGAGGTTT 3'
TrMt COIA	5' TGGGCATCCTGAGGTTTA 3'
TrMt COIB	5' GGACATAATGAAAATGAGC 3'
18S9 F	5' TGATCCTGCCAGTAGCATATGCTTG 3'
18S637R	5' TACGCTATTGGAGCTGGAGTTACCG 3'
CFor	5' ATGGCTCATTAAATCAGCTAT 3'
ARev	5' TGCTTTGAGCACTCAAATTTG 3'
MPLATCOX F	5' TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG 3'
MPLATCOX R	5' CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCAC C 3'

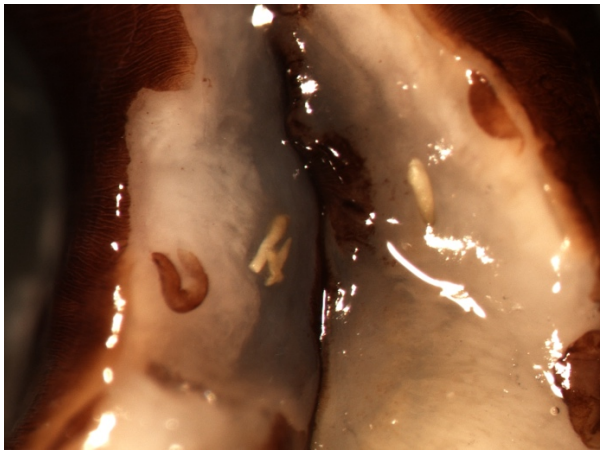
Table 2: primers set used for Trematodes

Each PCR was performed in a thermal cycler with a volume of 50 µl, containing 10 µl of 5X PCR buffer 1 µl of each dNTP, 0.5 µl Platinum SuperFi Taq Polymerase (invitrogen) 10 pmol primers, 1 µl of the extracted DNA, and water. The conditions set were: 94 ° C for 4 min, followed by 35 cycles of 94 ° C for 30 seconds, 60 ° C for 30 s and 72 ° C for 30 s and then 72 ° C for 7 min. The PCR products were visualized in 1% agarose gel and 800 bp size products were excised from the gel and purified using DNA, RNA and protein purification kits (Macherey-Nagel). The purified products were then sent for the sequencing service (Eurofin Genomics).

3. Results

3.1 Gross examination of the infected foot

Macroscopic identification of the parasite was carried out using a stereomicroscope. At the time of sampling the foot was isolated from the animal and cut in sagittal section to observe anomalies, lesions, presence of foreign bodies.



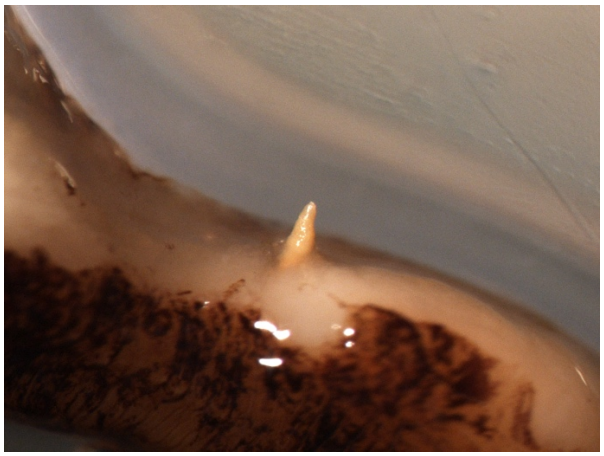


Figure 10: 823MYT8 (Bagnoli), occurrence of parasites in a mussel foot cut in sagittal section.
Figure 11: 848MYT11(Capo Miseno), parasite coming out of the tissue.

On the cutting surface it was possible to notice different types of lesions in the foot. The lesions to the presence of parasites (Figure 10, 11) were yellowish in color and variable in size and ovoid / elongated in shape and clearly distinguishable from the

surrounding muscle tissue. In some cases, the parasites emerged from the cut section, and it was possible to pull them out from the muscular tissue (Figure 11). Other lesions found at the stereomicroscope appeared as roundish delimited areas, pale compared to the surrounding tissue and noticeable both superficially, with a loss of the foot pigmentation in the affected area (Figure 12), or when cut, with disruption of the texture of the foot tissue and the occurrence of a rounded whitish area.

Figure 12: Nodule on the foot surface with loss in the epithelial pigmentation, 979MYT13 (Pozzuoli).

3.2 Animal histopathology

Through a retrospective review of the samplings carried out at the laboratory of Pathology of Marine Organisms of the Department of Biology, Federico II, we noticed that the occurrence of metacercariae in the foot of mussels has always been reported in several animals. (Table 3). The histological examination carried out in the past, however, considered only the presence or absence of the parasite and the infected animals could've been underestimated due to the preparation of the histological sections in which the foot is not always present, or not entirely included in the sections.

n.	Date	Area	Animals	Metacercariae	Prevalence
194	22/04/2008	Villaggio Coppola (CE)	20	2	10%
206	16/6/2008	Bagnoli	8	1	12,5%
534	25/3/2010	Castellamare	20	1	5%
550	14/6/2010	Capo Miseno	20	2	10%
576	10/5/2011	Castellamare	20	1	5%
581	22/9/2011	Capo Miseno	20	3	15%
594	1/3/2012	Capo Miseno	20	1	5%
763	31/07/2018	Torre Annunziata	30	3	10%

Table 3: Occurrence of metacercariae in mussel's foot in retrospective study (2008-2018)

During the sampling for the current study, we decided to isolate the foot and evaluate the presence of both macroscopic (as we mentioned in the previous section) and microscopic lesions. For the preparation of the histological slides, the foot was cut in sagittal section and embedded in paraffine in order to obtain a longitudinal section for viewing the foot in its entirety. Light microscopy allowed us to determine the main structures of the mussel's foot in a physiological condition and evaluate the lesions and the tropism of the parasite.
Foot microscopic anatomy was descripted according to previous studies (Fig 13): the foot presents a lining with a simple ciliated epithelium, the pedal musculature consists of two pairs of retractor muscles and a complex of fibres within the foot, pedal ganglia are fused in a dorsal position at the base of the foot, the glands can be divided into two main groups: pedal glands, marked by the letter P, and the stem glands, marked by the letter S. Glands S1 are located at the base of the foot in a medial position and their function is the formation of the primary byssus thread, S2 gland are situated between the posterior adductor and the gland S1, they contribute to the formation of the main structural component of the primary thread; glands P3 extend forward on each side of the pedal ganglia and converge at the midventral

pedal depression, those are deputed to the formation of the attachment plaques which cement the byssus to the substratum (Lane, & Nott, 1975).

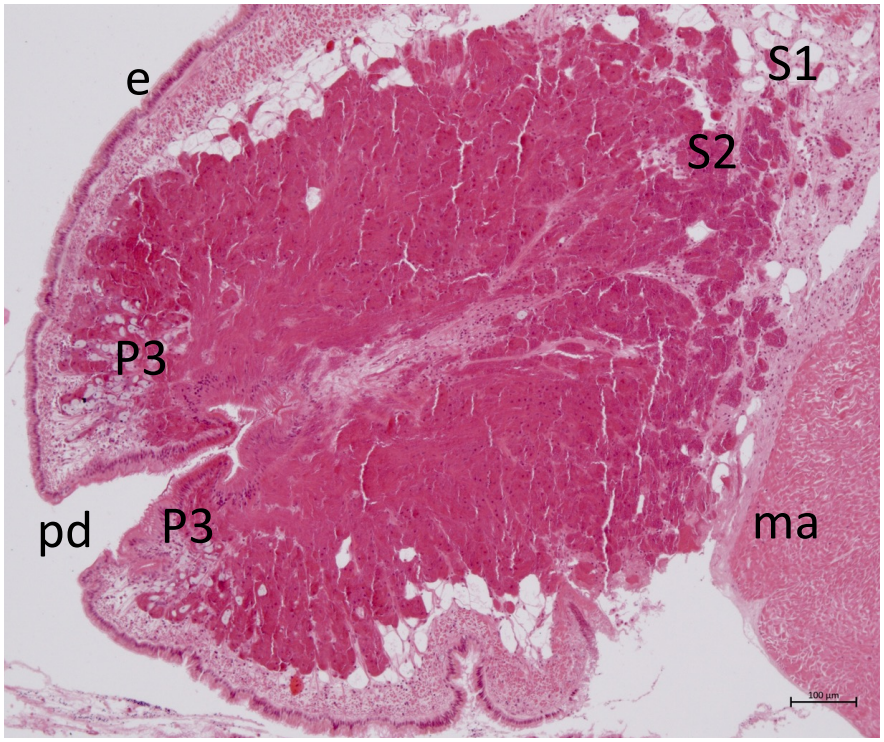


Figure 13: physiological foot anatomy: e: ciliated epithelia; pd: podal depression; ma: posterior adductor muscle; S1: glands of the byssus main thread; S2: glands of the structural component of the primary thread P3: formation of the attachment plaques. H&E.

The histological examination of the foot revealed the presence of the metacercariae in a variable number (1-8 per foot) and two other types of inflammatory lesions: cercariae and nodular lesions.

The lesions were variously distributed either in the byssus production glands (S1-S2) or the attachment plaque (P3), or in the muscular portion. (Fig 14 A-B)

The found metacercariae presented a PAS positive capsule, an oral an ventral sucker and an early development of reproductive organs (Fig 14 C-D)

The infestation was sometimes accompanied by a hemocyte infiltration in the podal artery, with small aggregates of inflammatory cells that infiltrated the glandular locations.

The inflammatory reaction was represented by flattened immune cells surrounding the parasites; in some cases, focal or diffuse hemocyte infiltration with central necrosis and degenerating hemocytes was observed.

The capsular inflammatory lesions consisted of granular hemocytes in the degranulation phase, which appeared flattened in order to circumscribe and eliminate the pathogen which in the early stages of cercaria appeared foamy, PAS positive.

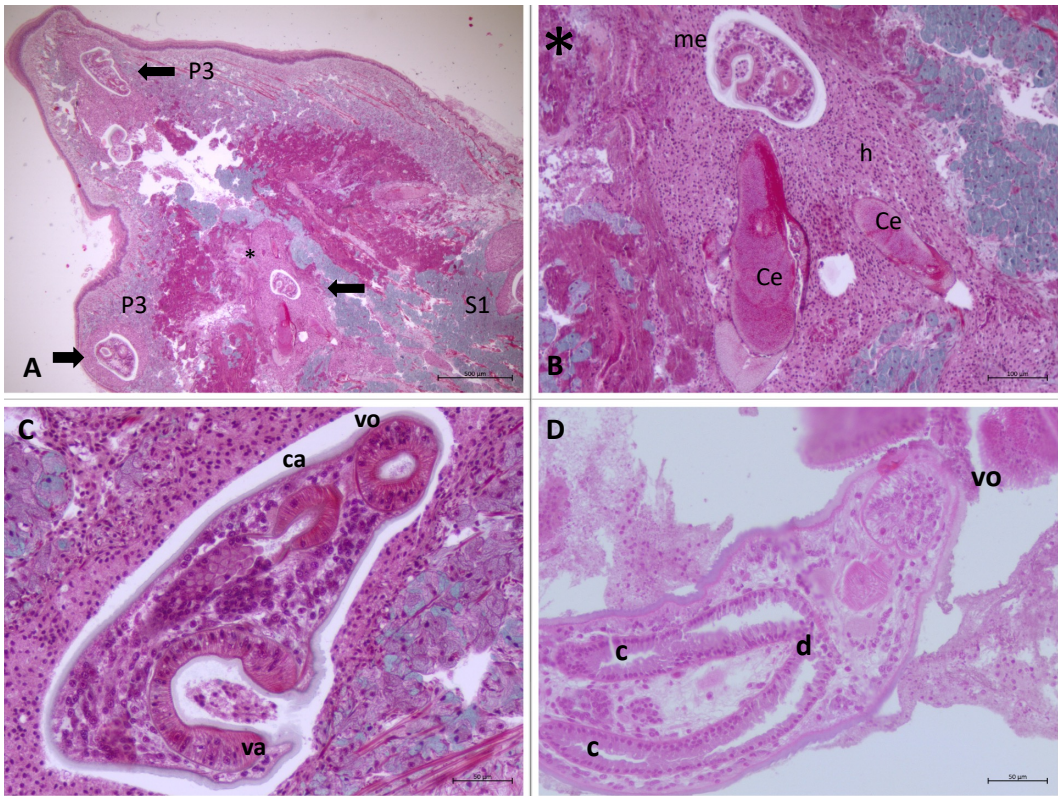


Figure 14. A) Mussel foot infested with multiple metacercariae (Arrows), in the muscular tissue; bissogenic glands (S1); attachment plaque glands (P3). Masson Trichrome. B): Detail of Fig 14-A: capsular inflammatory lesion consisting of hemocytes in the degranulation phase to eliminate the phlogogenic agent (me: metacercaria; h: hemocytes ; Ce: cercariae). Masson Trichrome. C): metacercaria phagocyting hemocytes (vo: oral sucker va: ventral sucker; ca: capsule). Masson Trichrome. D) Metacercaria in longitudinal section, visible the digestive system (d) with two ceca (c) H&E.

3.3 Disease distribution and effect

The overview of all the 323 examined samples since 2008 shows us that the parasite is always present in the mussels and the prevalence rate of metacercariae varies from 3.3% to 16.67% (Figure 15) with peaks in summer months (July / September).

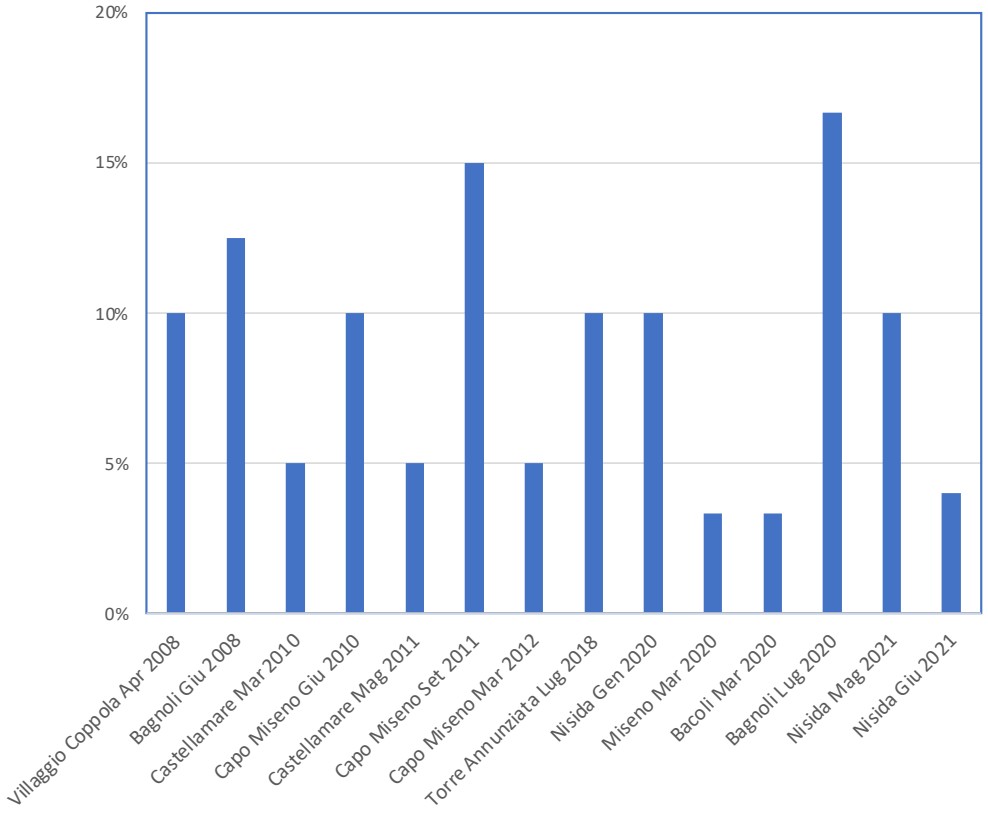


Figure 15: prevalence of metacercariae in the foot of mussels (2008-2021)

Histology of the entire foot allowed us to describe and report different typologies of lesions in mussel foot (Fig 16). The total number of analyzed samples since 2019 was 165 with a 7,4% of the animals infested with cercariae, 14,1% with nodular lesions and 5,2 % with visible Metacercariae.

Nodular inflammatory lesions, with no trace of the phlogogenic agent, were found in 26.6% of the animals, in particular in the sampling number 847 carried out in March 2020, there was only one metacercaria was found, while cercariae lesions

were detected in 13.3% of cases. In sampling 848 (March 2020) the prevalence of nodular lesions and cercariae in both cases was 5 out of 30 animals (16.67%). Finally, in sampling 849 (July 2020) the trend reverses with a higher prevalence of metacercariae (5/30) with less nodular lesions, found in only one animal, cercariae were found in 4 animals (13.3%) .

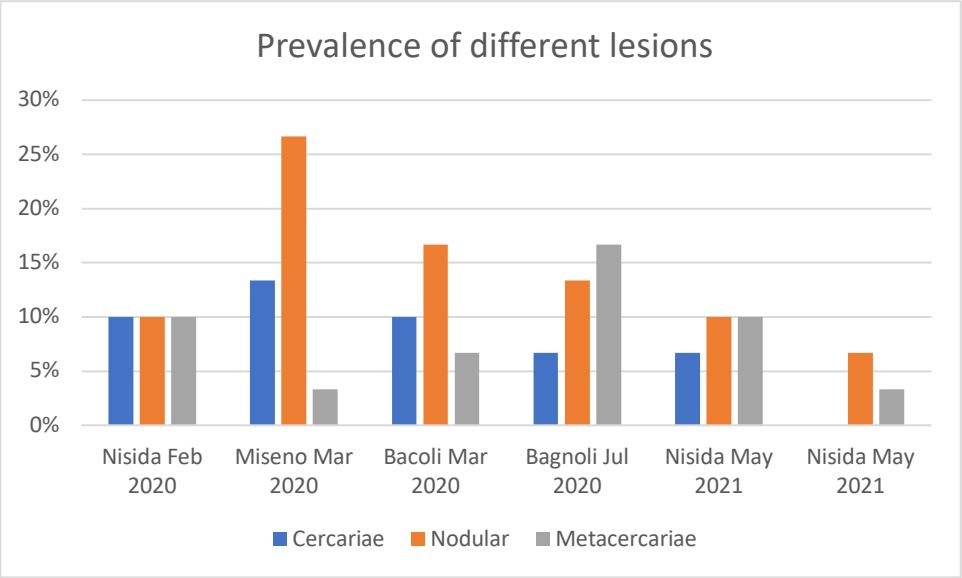


Figure 16: Prevalence in the samples of the different typologies of lesions.

3.4 Molecular diagnostics

Diagnostic carried out on the 18S rDNA show an identity of 94.2% in genebank with digenean trematodes of the genus *Opisthomonorcheides* (spp), parasites of the digestive tract of fish belonging to the Carangidae family, with reports mainly in Indonesian waters (Madhavi, 1977).

4. **Discussion**

Digenetic trematodes are the most frequent and most important metazoan parasites of bivalves (Cheng, 1967). Bivalve molluscs, similarly to terrestrial molluscs, represent intermediate hosts for marine trematodes, while final hosts are vertebrates (Lauckner, 1983) . Most reported trematode species affecting mussels *Mytilus sp.* include Fellostomidae (Villalba, 1997; Rayyan, 2004), Bucephalidae (Coustau, 1990), Gymnophallidae (Puljas, Burazin 2022; Marchiori 2023) and Echinostomatidae and Renicolidae (Nikolaev, 2006; Thieltges, 2006).

The present study is the first report of a digenean trematode of the Monorchidaee family in mussels and in the Mediterranean mussel *Mytilus galloprovincialis.*

The presence of metacercariae infecting the foot of *Mytilus galloprovincialis* was reported by Carella et al (2018) with an inflammatory capsule surrounding encysted metacercariae at mussel foot level. The present study investigated the systematic presence of this parasite infecting mussel foot. Our study show that this trematode was present in all sampled locations, moreover, isolating the foot from the rest of the visceral mass and dissecting it in a sagittal section allowed us to characterize better the trematode damage on the host. Gross examination of the whole foot revealed the presence of nodular type lesions on the external surface and the presence of cercariae in the muscle tissue on cut section. Histopathology of the sagittal section gave us a complete view of the mussels' foot with the possibility to determine the anatomy and the lesions caused by the trematode. Light microscopy showed the different inflammatory response of the host with the most frequent lesion characterized by the metacercariae cysts surrounded by an encapsulation-type response and nodular-type inflammation whereas the parasite was in phase of reabsorption and phagocytosed by the host (De Vico & Carella, 2012).

Encapsulation is a common immune response in mussels against foreign bodies such as parasites, the host starts enclosing the metacercaria when the circulating immunocytes interact with positively charged particles on the surface of the parasite starting the "recognition", moreover has been shown that opsonins with encapsulation-promoting activity are present in hemolymph (Jayaraj , 2009; Meena,

2010). Activated haemocytes interact with each other to form multicellular capsules to eliminate effectively the foreign intruder and in the end the cytotoxic products are released by heamocyte degranulation to "kill" the parasite. (Humphries and Yoshino, 2003; De Vico & Carella 2012). In the present study we reported in 14,1% of the cases the presence of nodular inflammatory reaction, meaning that the host successfully destroyed the parasite.

The mussel's foot is located in the center of the ventral margin of the visceral mass. Its function is the formation of the byssus filament and adhesive plaques that allow it to resist the wave motion and the action of predators. Cercarie of monorchidae contain penetrating secretory glands (Harada & Suguri, 2001) close to the ceca that may allow them to enter where the skin is thinnest and where byssus formation begins, the site where bacterial infections begin; otherwise cercariae can make their way in through the podal depression from which the byssus comes out. When metacercariae encyst in the byssus production glands, they carry out their parasite action by disrupting the physiological functioning of those glands, damaging indirectly the formation of the byssus or of the attachment plaques, in this way the efficiency of the mussel's adherence to the substrates is compromised making parasitized mussels more vulnerable to predation.

Molecular diagnostic of the metacercariae rDNA assigned the trematode to the genus *Opisthomonorcheides* Parukhin, 1996, family *Monorchiidae*, that comprehends 35 trematode species parasitizing fishes as final host (WoRMS, 2023). It has been reported by previous authors that in the Indo-Pacific areas definitive hosts of this trematode are represented by Carangidae, Stromateidae or Serranidae (Madhavi, 1977; Hafeezullah, 1984; Gupta, V. & Puri, 1984; Bray, 2017). Currently all the records in literature regard adult stages of this trematode, no description of *Opisthomonorcheides* metacercariae and intermediate hosts are reported. Most descriptions of the larval stages of digeneans in the family Monorchiidae come from bivalves found in the Atlantic and Pacific oceans. Along the Italian Tyrrhenian coast, metacercariae of Monorchidae belonging to the family *Postmonorchis* were detected in tissues of the Mediterranean wedge clam *Donax trunculus* (Carella , 2013), *Postmonorchis* infect several tissues including the foot. Other reports indicate the presence of Monorchidae of the genus *Postmonorchis* in

Italian Mediterranean sea in oysters (Mancini, 2018), indicating that digeneans parasitising oysters are commonly shared also with distantly related bivalve families (Lauckner 1983). This may suggest that *Opistomonorcheides* could also shift hosts in bivalves population and its presence and life cycle should be the object of further investigation.

To date there are no indication of how *Opisthomonorcheides* has been introduced in the Mediterranean sea and how this parasite complete its life cycle in this area. Further studies and molecular analysis are necessary to clarify the parasite species and life cycle. Identification of metacercariae based on morphological features is frequently challenging due to lack of specific characteristics in larval stages that could address the association with adult stages. Experimental infection and observation on fist and definitive hosts in the affected areas would allow to better clarify the life cycle and the recognition of species. Adults pahse is reported in Carangidae, a family of carnivorous fishes who feed also on mussels (Kulbicki, 2005). Carangidae of the species *Trachurus trachurus* and *Seriola dumerili* are common in the Mediterranean sea and along the Thyrrenian sea coast. Although in the Mediterranean Sea more species of *Carangidae* are reported, some of which are alien fish species introduced to the Mediterranean Sea via the Suez Canal, Gibraltar or in ballast water (Oral, 2010). The colonization of the Mediterranean Sea by Indo-Pacific and Red Sea species via the Suez Canal, known as Lessepsian migration, is an ongoing process that has considerably enriched biodiversity in the Mediterranean Sea. (Golani, 2006). Among alien species reported in the Mediterranean, from Indo-Pacific ocean, alien Carangidae *Decapterus russelli* was reported by Golani in 2006, this fish is a definitive host of *Opisthomonorcheides* and specifically by *O. decapteri* (Bray et al 2017). To date there is no evidence of the infection by *Opisthomonorcheides* in this species or other Carangidae in the Mediterranean sea, however we cannot exclude that the trematode adults could be infecting carangid fishes around mussel farms in the area of the study.

CONCLUSIONS

The expansion of aquaculture, including mussel farming, has been accompanied by the emergence of novel disease and the spread of known pathogens in new countries and seas.

In our study we reported the presence in *Mytilus galloprovincialis* of two pathogens, previously described in literature, with the an overview on the host-pathogen interaction and a first molecular description of both parasites. *Steinhausia mytilovum* has been often reported in the Mediterranean Sea and in populations of *M. galloprovincialis*, although in the present study we performed for the first time a molecular identification and classification of this microsporidium. *S. mytilovum* was reported over a decade infecting mussel's oocyte along the area of study, its impact on the host results in a loss of gonadal tissue and with a consequent loss in mussel weight. The consistent presence and circulation of *S. mytilovum* in farms located in the Gulf of Naples (especially in the areas of Capo Miseno and Bacoli where the microsporidium was found in all samplings) can lead to an economic loss for farmers, due to a lower weight in infected specimens. Further studies are needed to evaluate the progression of the infection by *S. mytilovum* and the life cycle of the microsporidium. Route of transmission should be investigated with studies on both vertical and horizontal transmission. Horizontal transmission can occur when spores are released with mature infected eggs or through phagocytosis and subsequent diapediasis (Jones and Creeper, 2006). The transgonadal or transovarian transmission has been described in various microsporidia (Durfort and Vallmitjana, 1982) and it has been also suggested for *S. mytilovum* (Maurand and Loubes 1979), in fact, presence of parasite vacuoles inside the host nucleus would facilitate the transgonadal transmission. The constant presence, prevalence and severity of *S. mytilovum* in the area may indirectly reflect immunocompetence at individual, population and ecosystems levels and need to be clarified. Further investigations should be carried out in order to verify the

transmission mechanism of *Steinhausia* and its potential host jumping to other bivalves or humans.

Regarding the trematode infection, its presence was already reported to infect mussel's foot but always considered as an occasional or minor pathogen. Sampling the whole foot of the mussel for macroscopic and histology has allowed us to assess the presence of the parasite, in some cases with an higher prevalence, but also to understand the host-pathogen interaction. Metacercariae were always present in all the sampled areas and the sagittal dissection of the foot allowed us to investigate further the presence of this parasite. We believe that the typologies of lesion identified in the foot denote the different stages of the parasite encystment in the tissue and the host reaction to the trematode. Metacercariae phase do not reproduce in the host, therefore are not as tissue destructive as sporocysts in first intermediate mollusc hosts (Lauckner, 1983). Although, combined with other stressors, trematodes may effect survival of their second intermediate host (Wedgeberg & Jensen, 1999; Lafferty & Kuris, 1999). Metacercariae and related lesion were mostly found close to the glandular system of mussel's foot. The parasite action on the host is translated in a lower capability of the mussel in the production of the byssus thread and the attachment plaques, which make infested mussels more vulnerable to predators. Further studies need to be carried in order to assess the life cycle, the definitive host and other primary or secondary intermediate hosts in the area. Molecular diagnostics results identified the trematode with *Opisthomonorcheides sp.*, with adult species reported only in Pacific and Indian ocean fishes. This is the first description in the Mediterranean Sea of this trematode, to date there is no data about how it entered this area and its life cycle.

Definitive hosts of *Opistomonorcheides* are well defined and are mainly represented by Carangidae (Bray, 2017), thus we cannot confirm that autochthonous fishes belonging to this family represent the definitive host in the Mediterranean area.

The study of *Steinhausia mytilovum* and *Opisthomonorcheides* sp. in the local populations of *Mytilus galloprovincialis* needs further research into the epidemiological effects of these molluscan pathogens on this economically significant species, to better characterize the infection impact, and the life cycle of

these two species and the potential risks of host-shift, and in the end to understand the surveillance necessary to prevent spread of the diseases in mussel populations.

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