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Occurrence, molecular identification, and *in vitro* features of emerging zoonotic parasites in Mediterranean marine fish

TESIS DOCTORAL

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Samantha Moratal Martínez

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Los abajo firmantes, como directores de esta Tesis Doctoral realizada por la doctoranda Dña. Samantha Moratal Martínez y titulada "**Occurrence, molecular identification, and *in vitro* features of emerging zoonotic parasites in Mediterranean marine fish**", hacen constar que reúne los requisitos necesarios para su defensa y aprobación y, por tanto, para optar al grado de doctor con mención internacional.

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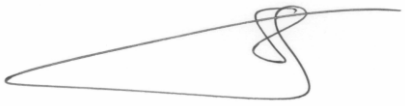

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ABSTRACT

Fish has core relevance in humans' diets all over the world, both for its importance in food security and for its enormous nutritional benefits. However, fish consumption is not exempt from risks, such as the presence of chemical pollutants or disease-causing biological agents, such as parasites. In particular, marine fish serve as hosts to a wide variety of parasites, including foodborne emerging zoonotic parasites, as nematodes of the genus *Anisakis*, of significant public health concern. Furthermore, they can also harbor other emerging zoonotic parasites originating from terrestrial environments, capable of reaching and contaminating marine waters. In both cases, the presence of these parasites in marine fish intended for human consumption represents a potential risk of transmission to humans. Given the distinct nature of these two groups of parasites and their varying research requirements, this doctoral thesis is structured into two parts.

The **first part**, consisting of two chapters, focused on the molecular detection and characterization of emerging unicellular zoonotic parasites of terrestrial origin in marine fish from the Spanish coast of the western Mediterranean. In the **first chapter**, different PCR techniques were employed to determine the prevalence of *Cryptosporidium* spp., *Blastocystis* sp., and zoonotic microsporidia species in the gastrointestinal tract of three groups of marine fish from the coast of the Comunidad Valenciana (total N = 408): aquaculture fish, wild fish from the surroundings of the aquaculture facilities, and wild fish from extractive fisheries. Zoonotic species and subtypes were detected for all three parasites/parasite groups under study, namely *Cryptosporidium ubiquitum*, *Blastocystis* sp. subtypes (STs) ST5, ST6, and ST7, and *Encephalitozoon hellem*/*Encephalitozoon intestinalis*. However, the prevalences were very low, suggesting a limited risk of transmission through fish consumption in the studied fish populations.

In the **second chapter**, a metabarcoding dietary approach based on the V8-V9 region of the 18S rRNA gene was used to investigate the parasite community present in digestive samples from two ecologically and economically important pelagic fish species in the western Mediterranean, the European pilchard (*Sardina pilchardus*)

and the round sardinella (*Sardinella aurita*) (total N = 47). Organisms belonging to four parasitic classes were identified, with higher significant prevalence in European pilchards for Cestoda and Myxozoa. Unicellular zoonotic parasites (*Cryptosporidium* sp., *Blastocystis* sp., and *Kudoa thyrsites*) were found in very low presence and number of reads. However, false negatives were detected for *Cryptosporidium* and *Blastocystis*. Therefore, this technique proved useful for exploratory studies of parasite communities in both species but presented limitations in terms of coverage, taxonomic depth, and detection of rare or low abundant organisms. The use of alternative primers and hierarchical metabarcoding is suggested as an improvement for future studies.

The **second part**, corresponding to the **third chapter**, focused on *Anisakis pegreffii*, the most relevant zoonotic parasite transmitted through fish consumption in the Mediterranean basin. The primary objective was to contribute to the development of the *in vitro* life cycle of *A. pegreffii*, crucial for understanding key aspects of the host-parasite interaction and for providing biological material for subsequent research. Therefore, the fecundity and fertility window of two populations of *in vitro* cultured adult *Anisakis* (*Anisakis simplex* sensu lato (s.l.) and *A. pegreffii*) were evaluated; a protocol to obtain third-stage larvae (L3, the infective stage) from eggs laid by *A. pegreffii* adults in culture was developed; ultrastructural characteristics of recently hatched second-stage larvae (L2) of *A. pegreffii* were studied; and chromosomal extensions were obtained for karyotyping from gonadal tissue of adult *A. pegreffii*. Results showed high average fecundity, yielding sufficient biological material (eggs and larvae) for additional experiments. The *in vitro* protocol successfully produced L3 larvae that were maintained in culture for up to 17 weeks. Ultrastructural examination revealed relevant features, including the presence of the excretory gland cell (of utmost relevance in *Anisakis* pathogenicity) in larvae as young as 48 hours old, as well as the release of extracellular vesicles and cell-free mitochondria. Finally, a diploid karyotype, potentially 2n = 18, was obtained.

RESUMEN

El pescado tiene una relevancia fundamental en la dieta de los seres humanos en todo el mundo, tanto por su importancia en la seguridad alimentaria como por sus enormes beneficios nutricionales. Sin embargo, el consumo de pescado no está exento de riesgos, como la presencia de contaminantes químicos o agentes biológicos causantes de enfermedades, como los parásitos. En particular, los peces marinos son hospedadores de una amplia variedad de parásitos, incluyendo parásitos zoonósicos emergentes de tansmisión alimentaria, como es el caso de los nematodos del género *Anisakis*, que representan una gran preocupación para la salud pública. Además, también pueden albergar otros parásitos zoonósicos emergentes que tienen su origen en el medio terrestre y que son capaces de alcanzar y contaminar las aguas marinas. En ambos casos, la presencia de estos parásitos en peces marinos destinados al consumo constituye un potencial riesgo de transmisión a los seres humanos. Dada la diferente naturaleza de estos dos grupos de parásitos y sus diferentes necesidades de investigación, la presente tesis doctoral se estructura en dos partes.

La **primera parte**, compuesta por dos capítulos, se enfocó en la detección y caracterización molecular de parásitos unicelulares zoonósicos emergentes de origen terrestre en peces marinos de la costa española del Mediterráneo occidental. En el **primer capítulo**, se utilizaron diferentes técnicas de PCR para determinar la prevalencia de *Crytposporidium* spp. *Blastocystis* sp. y especies zoonósicas de microsporidios en el tracto gastrointestinal de tres grupos de peces marinos de la costa de la Comunidad Valenciana (N total = 408): peces de acuicultura, peces silvestres de las immediaciones de las explotaciones de acuicultura y peces silvestres procedentes de pesca extractiva. Se detectaron especies y subtipos zoonósicos para los tres parásitos/grupos parasitarios objeto de estudio, a saber, *Cryptosporidium ubiquitum*, *Blastocystis* sp. subtipos (STs) ST5, ST6 y ST7, y *Encephalitozoon hellem*/*Encephalitozoon intestinalis*. Sin embargo, las prevalencias fueron muy bajas, lo que sugiere un riesgo limitado de transmisión por consumo de pescado en las poblaciones de peces estudiadas.

En el **segundo capítulo** se empleó un enfoque de metabarcodificación para dieta basado en la región V8-V9 del gen 18S rRNA para estudiar la comunidad de párasitos presente en muestras digestivas de dos especies de peces pelágicos de gran importancia ecológica y económica en el Mediterráneo occidental, la sardina Europea (*Sardina pilchardus*) y la alacha (*Sardinella aurita*) (N total = 47). Se identificaron organismos pertenecientes a cuatro clases parasitarias, con una prevalencia significativamente mayor en las sardinas para Cestoda y Myxozoa. Se detectaron parásitos zoonósicos unicelulares (*Cryptosporidium* sp., *Blastocystis* sp., y *Kudoa thyrsites*) en baja presencia y cantidad de lecturas. Sin embargo, se detectaron falsos negativos para *Cryptosporidium* y *Blastocystis*. Por lo tanto, esta técnica resultó útil para el estudio exploratorio de las comunidades parasitarias de ambas especies, pero presentó limitaciones en términos de cobertura, profundidad taxonómica y detección de organismos raros o poco abundantes. Se sugiere el empleo de otros primers y la metabarcodificación jerarquizada como mejora para futuros estudios.

La **segunda parte**, correspondiente con el **capítulo 3**, se centró en *Anisakis pegreffii*, el parásito zoonósico transmitido por consumo de pescado de mayor relevancia en salud pública en la cuenca Mediterránea. El objetivo principal fue contribuir al desarrollo del ciclo biológico *in vitro* de *A. pegreffii*, crucial para la comprensión de aspectos clave de la interacción hospedador-parásito y para proporcionar material biológico para investigaciones posteriores. Así pues, se evaluó la fecundidad y ventana de fertilidad de dos poblaciones de *Anisakis* adultos cultivados *in vitro* (*Anisakis simplex* sensu lato (s.l.) y *A. pegreffii*); se desarrolló un protocolo para obtener larvas de tercer estadio (L3, la fase infectante) a partir de huevos depositados por adultos en cultivo de *A. pegreffii*; se estudiaron las características ultrastructurales de las larvas de segundo estadio (L2) recién eclosionadas de *A. pegreffii*; y se obtuvieron extensiones cromosómicas para cariotipado a partir del tejido gonadal de adultos de *A. pegreffii.* Los resultados mostraron una elevada fecundidad media, dando lugar a suficiente material biológico (huevos y larvas) para experimentos adicionales. El protocolo *in vitro* logró producir larvas L3 que se

mantuvieron en cultivo hasta 17 semanas. El estudio ultraestructural reveló características relevantes, como la presencia de la célula de la glándula excretora (sitio de mayor relevancia en la patogenicidad de *Anisakis*) en larvas de tan solo 48 horas, o la descarga de vesículas extracelulares y mitocondrias libres. Por último, se obtuvo un cariotipo diploide, potencialmente 2n = 18.

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ºC	degree Celsius
µg	microgram
µL	microlitre
µM	micromolar
16S/18S rRNA	16S/18S ribosomoal ribonucleic acid
ADS	Asociación de Defensa Sanitaria
ASVs	Amplicon sequence variants
BLAST	Basic Local Alignment Search Tool
bp	base pairs
COI	cytochrome c oxidase subunit 1 gene
cox2	cytochrome c oxidase subunit 2 gene
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS	chicken serum
Ct	cycle threshold
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EGC	excretory gland cell
EIDs	(re)emerging infectious diseases
ESPs	excretory/secretory products
EVs	extracelular vesicles
EWUP	emerging waterborne unicellular parasites
FAO	Food and Agriculture Organization
FISH	fluorescence *in situ* hybridization
g	gram
GFCM	General Fisheries Commission for the Mediterranean
GP60	60-kDa glycoprotein gene
h	hour
ID	identity
ITS	internal transcribed spacer region gene
L	litre
L2	second-stage larva/e
L3	third-stage larva/e

L3P	large third-stage larva phenotype
L4	fourth-stage larva/e
M	molar
m	metre
mg	miligram
min	minute
mL	mililitre
ML	Maximum-Likelihood
mm	milimetre
mM	milimolar
mOsm	milliosmoles
NCBI	National Center for Biotechnology Information
ng	nanogram
NGS	next generation sequencing
NIAID	National Institute of Allergy and Infectious Diseases
OTUs	operational taxonomic units
p	*p*-value
PCR	polymerase chain reaction
pM	picomolar
pmol	picomol
P/S	penicillin/streptomycin
QC	query cover
RFLP	restriction fragment length polymorphism
s	second
S3P	small third-stage larva phenotype
SAR	Stramenopiles, Alveolata, and Rhizaria
SDGs	Sustainable Development Goals
SDM	Schneider's *Drosophila* medium
SE	standard error
s.l.	sensu lato
SNV	single nucleotide variant
s.s.	sensu stricto

SSC	sodium citrate buffer
SSS	sea salt solution
ST	subtype
STD	standard deviation
TEM	transmission electron microscopy
Tm	melting temperature
WHO	World Health Organization

INTRODUCTION

In an ever-evolving world with a burgeoning global population, the consumption of fish has emerged as a critical focal point within the realm of nutrition, food security, and environmental sustainability. However, fish is not without its risks. This thesis delves into emerging zoonotic parasites in Mediterranean edible marine fish. We are going to start by introducing some data on fish consumption and production, to continue with emerging zoonotic parasites specific to fish hosts, and finally, to document the potential presence of other waterborne zoonotic parasites in marine fish hosts. Note that data provided in the following subsections on fish consumption and production refer primarily to the years 2019 and 2020. This corresponds to the most recent public updates available from the Food and Agriculture Organization (FAO).

1. Overview on marine fish consumption

1.1. Consumption of aquatic products

Global consumption of aquatic food has increased at a mean annual rate of 3.0% in the last six decades, which means that we are now consuming five times more aquatic food than 60 years ago. This increase has been produced in parallel to the population growth (mean annual rate of 1.6%), but also because of an increase in per capita consumption, from 9.9 kg in the 1960s to 20.5 kg in 2019. However, per capita consumption is widely variable according to the region/country and is highly influenced by aquatic food availability, income status, or food habits. It is noteworthy that the per capita consumption annual growth rate is the highest in upper-middle-income countries, with the notable leadership of China (3.2%), compared to lower-middle-income countries (1.9%) or high-income countries (0.5%; high consumption already in previous decades). Finally, low-income countries are experiencing a 0.2% decrease through the years (**Figure 1**). Despite this, in several low-income countries with high levels of undernutrition, aquatic food protein contributes to a greater extent to the total animal protein intake, being a key factor in food security (FAO, 2022a).

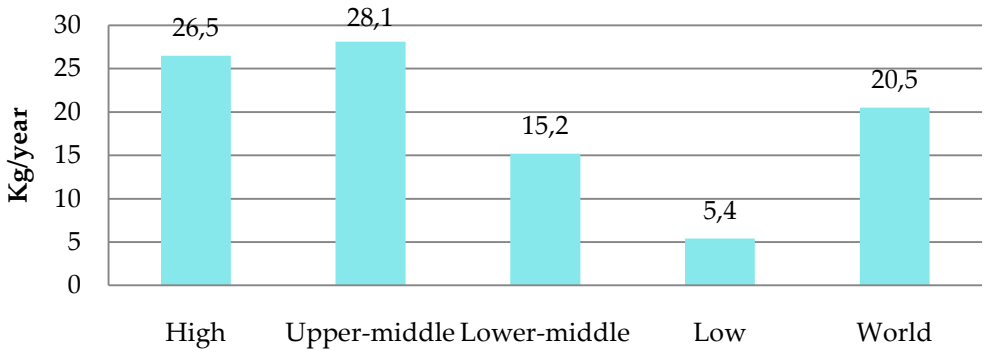

Figure 1. Aquatic food consumption (per capita consumption in kg/year) according to country income status. Year 2019. Adapted from FAO (2022a).

If considering the most recent available data (year 2019), from the total per capita intake, 75% came from finfish, the rest being shellfish. In turn, marine finfish accounted for the 33% (FAO, 2022a). It is worth mentioning the high rate of seafood consumption in the European Mediterranean countries, 30.86 kg per capita, compared to the global average of 20.5 kg in 2019. Interestingly, the leadership is held by Portugal (59.9 kg per capita) and Spain (46 kg per capita) (European Commission, Directorate-General for Maritime Affairs and Fisheries, 2022; FAO, 2022a).

1.2. Origin of edible marine-fish for human consumption

Seafood for human consumption is provided by capture fisheries production and aquaculture production. In the last decades, the increasing demand for seafood products has been possible to cover because of significant growth in the aquaculture sector, rather than capture fisheries, with stagnant productions (Carvalho and Guillen, 2021).

Interestingly, marine fish is the only group in which most production still comes from capture fisheries, with less than 10% coming from aquaculture. This is in contrast with other aquatic food groups (freshwater and diadromous fishes, crustaceans, molluscs, and others) whereby aquaculture production far exceeds capture fisheries production (FAO, 2022a).

1.2.1. Capture fisheries

After an increasing trend from the 1950s to the late 1980s, capture fisheries production has been maintained relatively stable, with interannual fluctuations (86-93 million tonnes per year on average). From those, catch in marine waters represented 87.3% in the 2018-2020 period (FAO, 2022a) (**Figure 2**).

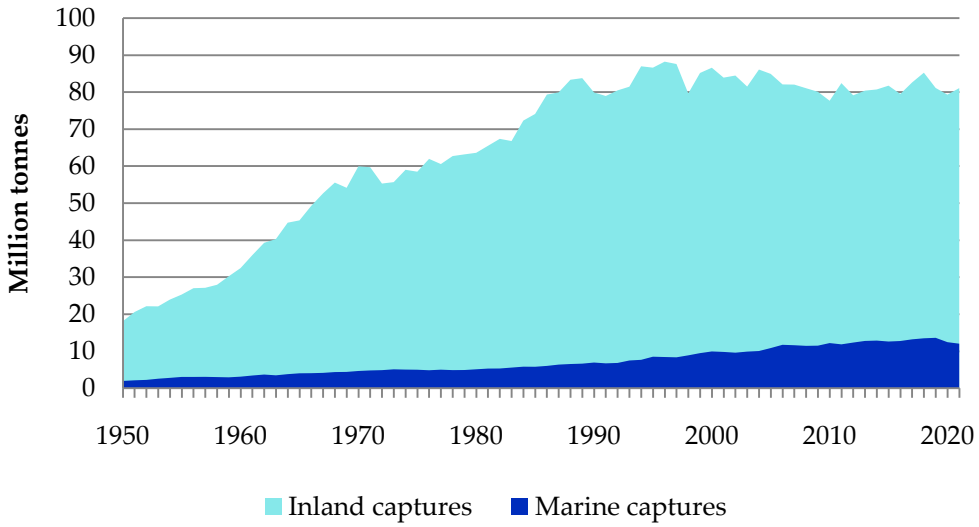

Figure 2. Temporal trends in global captures. Drawn from: FAO. Global capture production Quantity (1950-2021). License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/fishery/statistics-query/en/capture/capture_quantity. Data of Access: 19-07-2023.

Marine capture fisheries are dominated by a few producers, with the leading seven producers (China, Indonesia, Peru, the Russian Federation, the United States of America, India, and Vietnam) concentrating more than 50% of the captures (FAO, 2022a). The anchoveta (*Engraulis ringens*) heads the world marine finfish production, followed by the Alaska pollock (*Gadus chalcogrammus*) and the Skipjack tuna (*Katsuwonus pelamis*) (FAO, 2022a).

In contrast with the global stability in captures, the percentage of fishery stocks within biologically sustainable levels has decreased from 90% in 1974 to 64.6% in 2019, which means that at the present time, more than 35% of the stocks are overfished. Among FAO's major fishing areas, the Southeast Pacific (division 87)

and the Mediterranean and Black Sea (division 37) have the largest proportions of stocks being fished under unsustainable levels (66.7 and 63.4%, respectively) (FAO, 2022a).

In the Mediterranean Sea, capture fisheries production increased from 1970 until 1994, reaching a maximum of 1,087,100 tonnes. Since then, the production has gone irregularly declining, with the highest drop registered in 2020 (674,500 tonnes), probably stressed by COVID-19 restrictions (FAO, 2022b) (**Figure 3**).

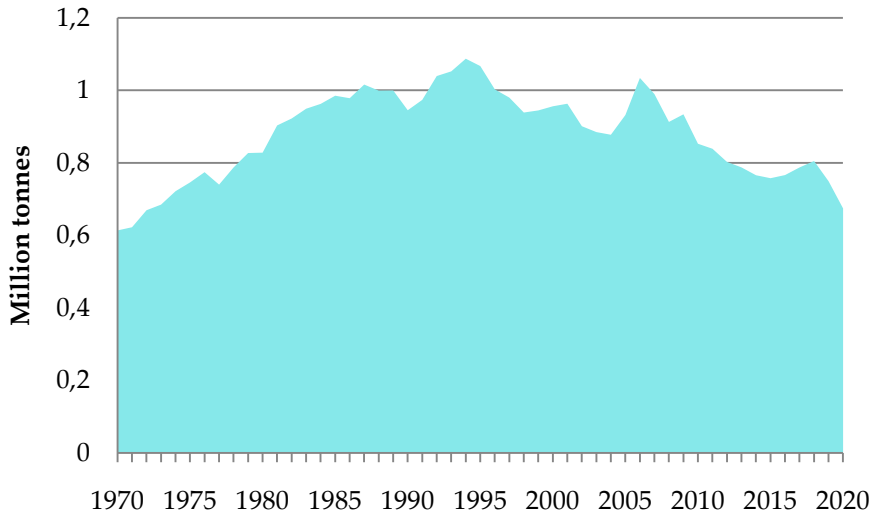

Figure 3. Temporal trends in Mediterranean captures. Drawn from: FAO. GFCM (Mediterranean and Black Sea) capture production Quantity (1970 - 2021). License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/fishery/statistics-query/en/gfcm_capture/gfcm_capture_quantity. Data of Access: 19-07-2023.

Based on last records (2018-2020 average), Italy is the main producer (20.9%) in the Mediterranean basin, followed by Tunisia (12.9%), Algeria (12.8%), Greece (9.9%), and Spain (9.5%). The small pelagic species, mainly herrings, sardines, and anchovies, contribute for slightly more than half of the total landings (FAO, 2022b).

Considering the General Fisheries Commission for the Mediterranean (GFCM) subregions, the western Mediterranean is the most productive subregion (20.3% of

total Mediterranean landings), with Algeria being the main producer (39.5%), followed by Spain (29.2%) and Italy (16.3%). Small pelagic species, mainly pilchard (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), and sardinellas (*Sardinella* spp.) are again the main caught species (FAO, 2022b).

1.2.2. Marine finfish aquaculture

Due to capture fisheries stagnation, aquaculture production has led the growth of total seafood production since the 1980s. Farmed finfish have represented the largest proportion of aquaculture production for decades (excluding algae). In particular, of the total 122.6 million tonnes of aquaculture production in 2020, 57.5 million tonnes were farmed finfish, and from these, 8.3 million tonnes were from mariculture and coastal aquaculture (**Figure 4**). The Atlantic salmon (*Salmo salar*), followed by the milkfish (*Chanos chanos*), stands out among the overall mariculture and coastal finfish species produced globally. They account for 32.6% and 14%, respectively, of the total production within this group in 2020 (FAO, 2022a).

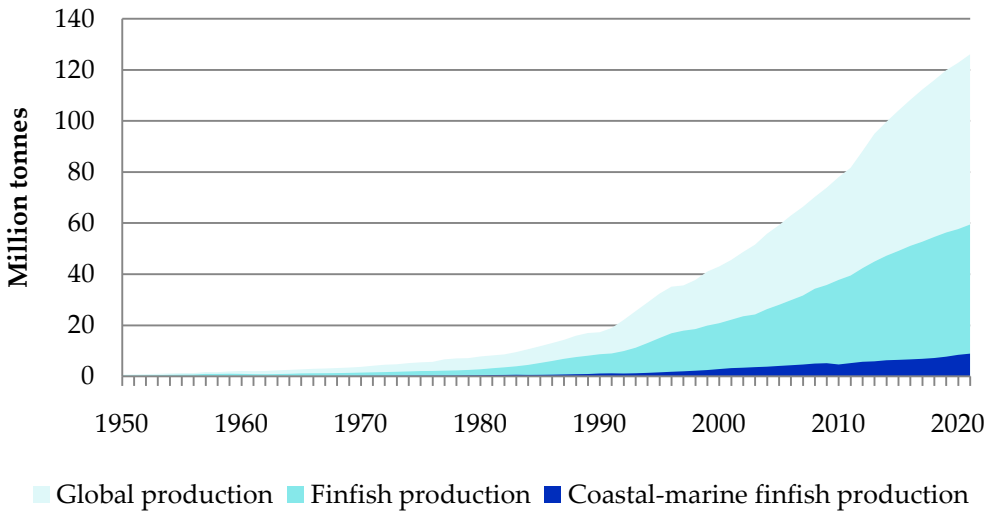

Figure 4. Temporal trends in world aquaculture production. Drawn from: FAO. Global aquaculture production Quantity (1950 - 2021). License: CC BY-NC-SA 3.0 IGO. Extracted from: https://www.fao.org/fishery/statistics-query/en/aquaculture/aquaculture_quantity. Data of Access: 19-07-2023.

Finfish mariculture and coastal aquaculture are led by Asia, with Europe in the

second position. In Europe, two types of regions/productions can be considered: the predominant marine cold water production (northern Europe, mainly Norway), dominated by the Atlantic salmon, and the marine Mediterranean production, mastered by the European seabass (*Dicentrarchus labrax*) and the gilthead seabream (*Sparus aurata*). The main producing countries in the Mediterranean are Turkey, Greece, and Spain (FEAP, 2022).

Although the general situation is that most of the marine fish still comes from capture fisheries, the situation in the Mediterranean basin is different. In the Mediterranean, capture fisheries are still predominant, but aquaculture production has experienced a 15% increase in the last ten years, reaching 43% of the total Mediterranean production in 2019 (Carvalho and Guillen, 2021).

1.3. Benefits and risks from fish consumption

Fish are an important source of high-quality protein. This fact is especially relevant in low-income countries, where aquatic food protein is the main source of animal protein, therefore having an important role in food security (FAO, 2022a). Indeed, The Sustainable Development Goals (SDGs) of the United Nations include food security as a priority, as well as marine resources conservation and sustainability, as stated in SDGs 2 and 14: "*End hunger, achieve food security and improved nutrition and promote sustainable agriculture*", and "*Conserve and sustainably use the oceans, seas and marine resources*", respectively. Fisheries and aquaculture have been only explicitly, but superficially mentioned, in these both goals. However, several authors pointed out that fish, and especially aquaculture, could constitute a key factor in achieving the overall 17 SDGs, including those that are directly related, such as SDG 2 and 14, but also others with indirect or associate relationships (Troell et al., 2023).

Beyond that, fish consumption has several nutritional benefits and impacts positively on human health. Fish is rich in several essential nutrients, including n-3 polyunsaturated fatty acids, vitamins (especially A, B, and D), essential amino acids, and minerals, that are known to have interesting properties for human health, like

anti-inflammatory, anti-carcinogenic or antioxidant activity (Chen et al., 2022). The synergistic effect of these activities, among several others, is thought to be behind the protective associations found between fish consumption and several health outcomes. Particularly, fish consumption has been linked to a reduced risk of mortality; it also decreases the risk of several cancers (brain, hepatocellular, and lung cancer, among others), cardiovascular and ischemic diseases (acute coronary syndrome, cerebrovascular disease or stroke), cognitive diseases (like depression or dementia risk), and even allergic condition (Zhao et al., 2023). Therefore, fish have an important role as part of a healthy diet, with a recommended intake of at least two servings (≈ 240 g) per week, including one portion of oily fish (Piepoli et al., 2016).

However, eating fish, and seafood in general, can also pose health risks for consumers. Fish are a source of exposure to different chemical pollutants that they bioaccumulate from the environment or through food, including toxic metals and organic compounds (like polychlorinated biphenyls or organochlorine pesticides, among others), with known harmful effects on human health (Perelló et al., 2015; González et al., 2019). Moreover, fish also carry a plethora of biological agents, including viruses, bacteria, and parasites, that are responsible of foodborne diseases worldwide (Ziarati et al., 2022).

Due to the remarkable importance that fish has in worldwide diets, the assessment of the associated risks must be mandatory in both legislative and scientific fields. Hereafter, we focus on emerging parasites responsible for fishborne diseases.

2. Emerging marine fishborne parasitic zoonoses

2.1. The concept of emerging parasitic zoonoses

According to the World Health Organization (WHO), emerging and re-emerging infectious diseases (EIDs) concepts refer to a group of diseases that have originated in recent years or that have already existed for a long time but are experiencing

rapid increase in incidence or changing their geographic distribution. In several cases, EIDs are zoonotic, therefore transmitted from animals to humans through direct contact or by food, water, or environment, including vector-borne diseases.

The emergence of the infectious agents involved in EIDs is driven by different factors that either involve greater contact between people and unusual pathogens or their natural hosts or foster their dissemination. These emergence drivers are usually linked to anthropogenic factors, although they can sometimes be due to natural causes, and can be classified as follows (adapted from Church, 2004; McArthur, 2019):

- Microbial adaptation, including the development of resistance to drugs, especially antibiotic resistance, or viruses' antigenic shifts.
- Ecological changes, mainly related to changes in the use of land and to climate change. Different human practices concerning land use, e.g., urbanization, deforestation, agricultural practices, construction of dams, etc., ultimately lead to close contact between humans and animals or arthropod-vectors, increasing the risk of exposure to infectious agents. On the other hand, climate change can impact the geographic distribution of hosts, but mainly arthropod-vectors, contributing to expanding their spatial ranges.
- Human demography: population growth and increased density of the human population, together with demographic movements (migration to urban areas, immigration) contribute to increasing the probability of getting in contact with new pathogens and/or facilitating their spreading.
- Technology, healthcare, and susceptibility to infection: especially in industrialized countries, technology has led to aging populations, sometimes suffering from chronic diseases. These population groups usually have impaired immune systems, making them more susceptible to infections.
- Breakdown in public health measures: conversely to industrialised countries, in developing countries, immunocompetence is hampered by malnutrition and the absence of immunization practice. Moreover, the lack of public

health infrastructure, the lack of access to safe water, and, in general, the poor sanitary conditions, enhance the probability of contact with pathogens.

- International travel and global trade, which enables people and animals, but also food products and other goods, to travel quickly around the world and therefore, to facilitate the spread of pathogens and their vectors.

Agents involved in zoonotic EIDs are bacteria, viruses, parasites, and fungi. However, the utmost attention and recognition is received by viruses and bacterial diseases. The National Institute of Allergy and Infectious Diseases (NIAID), lists the emerging infectious diseases/pathogens in three priority categories, according to their public health risk. Curiously, parasites received only one mention, corresponding to food and waterborne protozoa, in Category B. Also Microsporidia are listed in Category B; although they are currently classified as fungi (Adl et al., 2019), they are generally considered parasitic fungi. Two other parasites are listed as "*Additional emerging infectious diseases/pathogens*", namely acanthamebiasis and atypical *Babesia* (NIAID, 2018).

Traditionally, parasitic diseases had been related to poverty and considered to affect only underdeveloped and developing countries with low incomes (Chai et al., 2005). Therefore, little effort and resources have been invested in their study, concerning surveillance, prevention, and treatment. Paradoxically, the absence of good data on how these diseases impact health and economics makes it difficult to obtain more attention and resources. This state of neglect, together with the factors listed above, had provided a unique opportunity for several parasitic diseases to (re)emerge (Shamsi, 2019).

2.2. Diversity of emerging marine fishborne parasites

Within fish, the situation is even worse, with zoonotic parasites from fish receiving little attention compared to other well-known zoonotic parasitosis (Chai et al., 2005). Despite the high frequencies of zoonotic parasites detected in fish, especially in wild-caught fish, human infection cases are miss-recognized and totally

underdiagnosed (Shamsi, 2020). Among parasites, foodborne helminth parasites are those of great concern. While some of them are rare, others are known to cause severe illness and to represent a high risk to public health. This risk has increased in parallel to the rise in fish production, especially aquaculture, and the increment in seafood consumption. Moreover, changes in dietary habits, with growing interest in western countries in raw and undercooked fish dishes (sushi, sashimi, ceviche, etc.), together with climate change and the growth of international trading of fish and fish products, among other factors, have led to the emergence of several fishborne parasitic diseases (Lima dos Santos and Howgate, 2011; Fiorenza et al., 2020).

There are three major groups of zoonotic parasites infecting freshwater, brackish, or marine fish: trematodes, nematodes, and cestodes (Chai et al., 2005). People get infected by the consumption of raw or undercooked fish or fish products. Fishborne parasites have been reported worldwide, although their prevalence is notably high in Asian countries (Cong and Elsheikha, 2021), where the total and per capita aquatic food consumption are the highest worldwide (FAO, 2022a) and raw fish dishes are typically consumed.

2.2.1. Trematodes

Trematodes, commonly known as flukes, are the most diverse group of fishborne parasites and are considered emerging infectious pathogens (Ziarati et al., 2022). They are classified into three groups: liver, intestinal, and lung flukes. The general life cycle involves a definitive host (man or other piscivorous animals) and two intermediate hosts, a snail and a fish. The definitive host harbors the adult trematodes and sheds the embryonated eggs in feces. The eggs containing the miracidium may be directly ingested by a snail -the first intermediate host- or alternatively, the eggs may hatch and release the miracidium, which then penetrates the snail. After developing, the motile cercaria are released into the water, and subsequently, they find and penetrate the muscle of the second intermediate host, the fish, where they then develop into the infective stage, the metacercariae. Finally,

the definitive host acquires the infection when they ingest infected raw or undercooked fish (Klimpel et al., 2019).

Liver flukes, belonging to the family Opistorchiidae, are among the most prevalent trematodes in humans, being endemic in southern Asia. They are known to cause severe diseases, and even some of them are considered to be carcinogenic (IARC, 2012). It is noteworthy that about 45 million people are currently infected by liver trematodes, with more than 680 million at risk of infection (Saijuntha et al., 2021). However, they are only transmitted by freshwater fishes and therefore are out of the scope of this research. Similarly, pulmonary paragonimiasis (genus *Paragonimus*) is exclusively transmitted by freshwater crabs and crayfish (Shamsi, 2019).

Intestinal flukes are a diverse group with at least 74 zoonotic species worldwide distributed, highlighting heterophyds (Hetrophydae) and echinostomes (Echinostomatidae). It is estimated that around 40-50 million people are infected worldwide (Chai et al., 2009). Clinical manifestations are generally related to the gastrointestinal system, being more severe in echinostomes than in heterophyds infections. Intestinal flukes have life cycles occurring mainly in freshwater, but also in brackish water hosts (Chai and Jung, 2020).

2.2.2. Cestodes

Fish tapeworms (class Cestoda) belonging to Diphyllobothriidea family are the causative agents of human diphyllobothriosis (and diplogonoporosis). There are about 15 species reported to infect humans (Scholz and Kuchta, 2016), although only four of them have been studied in detail and are responsible for the majority of human cases, namely *Diphyllobothrium latum*, *Diphyllobothrium nihonkaiense*, *Diphyllobothrium dendriticum*, and *Adenocephalus pacificus* (Scholz et al., 2009; Kuchta et al., 2015a). Clinical signs of diphyllobothriosis are usually mild and not life-threatening, therefore arousing little attention. This fact, together with a better understanding of the different species distribution enabled by molecular tools, has set up conditions for the disease (re)emergence (Scholz and Kuchta, 2016).

D. latum and *D. nihonkaiense* are the first and second most-frequent fishborne tapeworms, with a total of 10 to 20 million estimated global cases and more than 2,000, respectively. Both, together with *D. dendriticum* (≈ 1,000 cases) are freshwater species, with freshwater fish and salmonids as second intermediate hosts (reviewed in Scholz and Kuchta, 2016). The knowledge of fish tapeworms' life cycle relies mostly on the well-studied, freshwater species. For marine species, marine mammals are known to be the definitive hosts; therefore it is assumed that marine copepods serve as the first intermediate hosts and marine fishes as the second intermediate hosts (Kuchta et al., 2015b). Humans get the infection when they eat raw or underprocessed fish or fish products that harbor the infective plerocercoids.

Among marine tapeworms, *A. pacificus* and *Diplogonoporus balanopterae* must be highlighted because of their importance in public health. *A. pacificus* (formerly known as *Diphyllobothrium pacificum*) is a parasite of otariids (fur seals and sea lions), of which twelve families of marine fish are known to be second intermediate hosts (Scholz and Kuchta, 2016). It has been reported from humans (and also dogs) who feed on raw or undercooked marine fish (Kuchta et al., 2015b), with approximately 1,000 cases in the last seven decades (Kuchta et al., 2015a). It is widely distributed in its host type, the otariids (Hernández-Orts et al., 2015), but human cases are mostly reported from the Pacific coast of South America, with special mention to Peru. However, five recent cases detected in Spain, focus on its potential expansion probably linked to international trading of marine fresh fish (transported on ice) from endemic areas (Kuchta et al., 2014; Pastor-Valle et al., 2014). *D. balanopterae,* the causative agent of the diplogonoporosis, is another cosmopolitan marine fish tapeworm, from different whale species. Japanese anchovy (*Engraulis japonica*), Japanese sardine (*Sardinops melanostictus*), and skip-jack tuna (*Katsuwonus pelamis*) are the recognized second intermediate hosts (Kino et al., 2002; Kuchta et al., 2015b). Around 300 cases of human infection have been recorded, the vast majority from Japan (Scholz and Kuchta, 2016). It is remarkable that four out of six cases diagnosed out of Japan were from Spain (Clavel et al., 1997;

Pastor-Valle et al., 2014).

2.2.3. Nematodes

Few fish nematodes are considered to cause emerging zoonoses; those that pose a risk to human health are anisakids (Anisakidae family) and members of Gnathostomatidae (*Gnathostoma* and *Echincephalus* genera).

The latter, just briefly mentioned, caused the so-called gnathostomiasis, linked to the consumption of raw or undercooked brackish or freshwater fish and amphibians and mainly reported in Southeast Asia, Central and South America (Herman and Chiodini, 2009). It produces allergic reactions and clinical disease that presents with nausea, vomiting, and abdominal pain. Larval subcutaneous migration produces inflammatory swelling (cutaneous disease) and may result in the penetration of other tissues and viscera (visceral disease) with more severe pathology; although rare, even the affection of the nervous system can occur, being fatal (Herman and Chiodini, 2009).

Family Anisakidae (Nematoda, Rhabditida) comprises eight parasitic genera of marine nematodes: *Anisakis* spp., *Contracaecum* spp., *Mawsonascaris* spp., *Phocascaris* spp., *Pseudoterranova* spp., *Pulchrascaris* spp, *Terranova* spp., and *Sulcascaris* spp. From those, members of the genera *Anisakis*, *Contracaecum*, and *Pseudoterranova*, included species that are zoonotic, causing the disease known as anisakidosis (Ángeles-Hernández et al., 2020). The specific term anisakiosis refers to the pathology caused only by members of *Anisakis* genus, mainly *Anisakis simplex* sensu lato (s.l.), which comprises, *Anisakis simplex* sensu stricto (s.s.) and *Anisakis pegreffii*, and, very rarely, *Aniskis physeteris*. These species are responsible for the vast majority of clinical cases caused by anisakids (Adroher-Auroux and Benítez-Rodríguez, 2020).

Anisakis spp., and anisakiosis, are explained in depth in the upcoming specific section for the following reasons, according to this thesis topic: (1) are cosmopolite

parasites, with worldwide distribution; (2) it is the only marine fishborne parasite genus that includes species present in the Mediterranean basin, with autochthonous human cases in Mediterranean countries; and (3) it is considered to be an emerging disease of great concern worldwide among those transmitted through fish consumption (Mattiucci et al., 2013; Ángeles-Hernández et al., 2020).

2.3. *Anisakis* spp.

2.3.1. Life cycle

Anisakis spp. has a complex life cycle, involving several hosts. Reproductive adults are located in the gastric chambers of marine mammals (Ángeles-Hernández et al., 2020). Eggs are eliminated with feces into the seawater, where they embryonate. Some authors consider that the stage which emerges from the egg is the second-stage larva (L2), while others consider it is already the third-stage larva (L3) still maintaining the L2 sheat (Køie et al., 1995; Mladineo et al., 2023). These free-swimming larvae are ingested by the first intermediate host, mainly euphausiids, pass through their digestive system, lose the L2 sheat and establish in the coelom cavity. Here, L3 larvae grow until reaching the infective size (Aibinu et al., 2019). The second intermediate host, generally considered a paratenic host, is fish or squid that prey on these infected euphausiids. Here, larvae are thought to be in paratenesis (Trumbić et al., 2021) until reaching the definitive host. Marine mammals acquire the infection when feeding on these paratenic hosts. Finally, in the definitive host, the L3 experiments two moults until reaching the adult stage (Aibinu et al., 2019). Accidental transmission to humans occurs when eating raw or undercooked seafood (Ahmed et al., 2016) (**Figure 5**).

2.3.2. Epidemiology

As stated above, the majority of diagnosed cases are caused by sibling species of the *A. simplex* s.l. complex, i.e., *A. simplex* and *A. pregreffii.* However, most of the clinical diagnoses rely only on morphological, sometimes histopathological, identification,

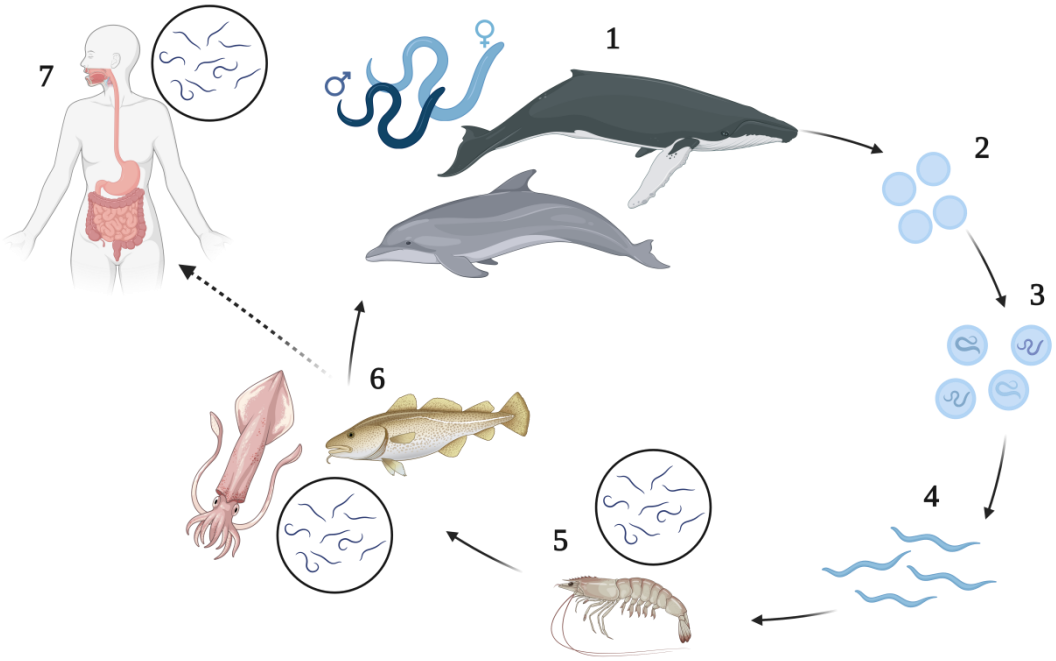

Figure 5. *Anisakis* spp. life cycle scheme. 1) The definitive host (marine mammal) ingests the paratenic host harboring the infective L3, which develop into reproductive adults. 2) Unembryonated eggs are released with the feces. 3) Eggs embryonate in water (L1-L2/L3). 4) Eggs hatch releasing the free-swimming L2/L3. 5) L2/L3 are ingested by the first intermediate host (euphausiid) and develop in the coelom. 6) The euphausiid containing the infective L3 is preyed by a paratenic host (fish or squid). L3 migrate and remain infective in its tissues. 7) Accidental transmission to humans. L3 cannot further develop. Created with BioRender.com.

with cannot provide accurate differentiation between different species from *Anisakis* genus. Only in 7% of the reported cases it has been specified that somewhat molecular identification was performed (Shamsi and Barton, 2023). Therefore, clinical cases are usually attributed to *Anisakis* spp., in general.

Humans get the infection through the consumption of raw or undercooked marine fish and squid (Ahmed et al., 2016). Several marine fish species have been identified to be hosts for *Anisakis* spp., indicating the low specificity of these parasites (Rahmati et al., 2020). Epidemiological studies provide important information that can be used in risk assessment, concerning the most common fishes affected in each geographical area, the prevalences detected on them, and the *Anisakis* species identified. The comprehensive review conducted by Rahmati et al. (2020) found that

Anisakis larvae were more prevalent in fish from five families, namely Lophiidae, Trichiuridae, Zeidae, Merlucciidae, and Gadidae. These authors also assessed the geographical risk, identifying the following high-risk regions: North and Northeast Atlantic Ocean, Southwest of the USA, West of Mexico, South of Chile, East of Argentina, Norway, UK, and West of Iceland (99% confidence) (**Figure 6**).

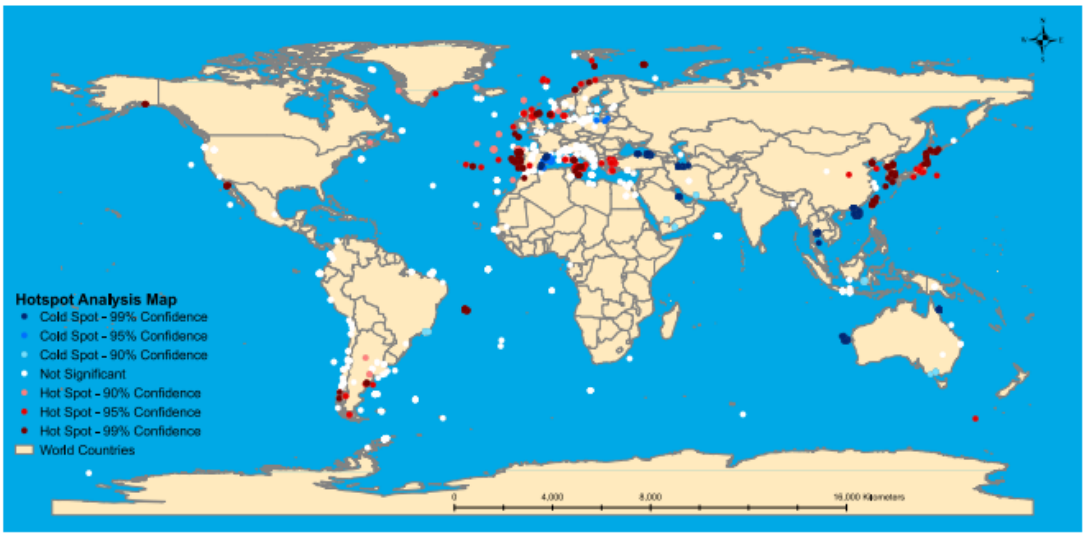

Figure 6. High-risk hot spots for *Anisakis* spp. in fishes. Source: Rahmati et al. (2020).

Concerning *Anisakis* species geographical distribution (both in definitive and intermediate hosts), *A. simplex* s.s. and *A. pegreffii* show different patterns. *A. simplex* s.s. is the most predominant species in the North Atlantic and North Pacific regions, while *A. pegreffii* is predominant in central and southern regions, highlighting the Mediterranean Sea. They are overlapping in some areas, like on the Northeast Atlantic coast of the Iberian Peninsula where coinfection occurs (**Figure 7**). Hybrids of both species have been largely detected, too (Mattiucci et al., 2018).

Number of reported cases of human anisakiosis is higher in countries with high per capita fish consumption and with culinary habits involving typical dishes based on raw or undercooked fish (Puente et al., 2008). Japan is the most affected country, followed by Spain, South Korea, Italy, and the USA (Shamsi and Barton, 2023). However, some authors agree on that anisakiosis is infra-diagnosed (Shamsi and Sheorey, 2018; Seal et al., 2020), as predictive models for risk assessment seem to

support. For example, in Spain, the number of annually reported cases is over 150 (Herrador et al., 2019), while a predictive model based only on anchovies consumption in the same country estimated 8,000 cases/year (Bao et al., 2017). Anisakiosis mimics the symptomatology of other gastrointestinal pathologies. This fact, together with the lack of formation among medical professionals in this parasitic disease, leads them not to include it in the possible differential diagnosis; this results not only in underdiagnoses but also in misdiagnosis (Moschella et al., 2004; Ramos et al., 2005; Shibata et al., 2014; Shamsi and Barton, 2023).

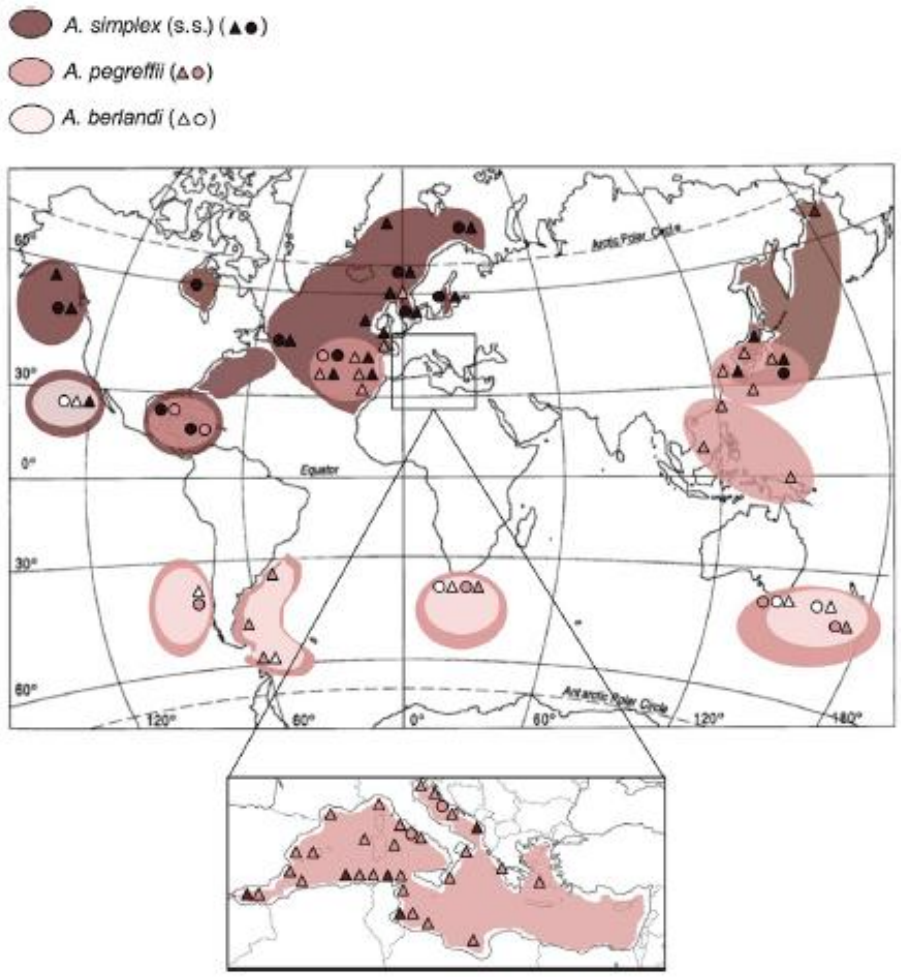

Figure 7. *Anisakis* species geographical distribution in the definitive hosts (circles) and intermediate/paratenic hosts (triangles). Source: Mattiucci et al. (2018).

2.3.3. Anisakiosis

Humans act as accidental hosts for *Anisakis* spp. The clinical illness results from the tissue damage caused by the ingested larvae when they attach to the gastric and occasionally intestinal mucosa. Different clinical manifestations have been reported (Adroher-Auroux and Benítez-Rodríguez, 2020):

- Gastric: is the most common presentation of anisakiosis and it is due to the larval attachment to the gastroduodenal mucosa. The onset takes place after two to six hours post-ingestion of the larvae and runs with epigastric pain, nausea, vomiting, diarrhoea, and urticaria. Symptoms remain while larvae are still alive.
- Intestinal: in this presentation, symptoms do not appear until day two or three and it is characterized by severe abdominal pain that can be accompanied by nausea, vomiting, or diarrhoea. Occasionally it can become chronic, with the formation of granulomas or abscesses.
- Extraintestinal anisakiosis, although rare, can occur if larvae manage to pass through the gastric wall and migrate to different organs causing specific symptomatology.

On the other side, gastroallergic anisakiosis is a recognized entity consisting of an acute IgE-mediated allergic response, characterized by urticaria, angioedema, or even severe anaphylaxis, that arises about 24 h after acute infection with live larvae and that is usually accompanied by mild gastric or abdominal symptoms (Daschner and Pascual, 2005). In addition, allergic anisakiosis associated with *Anisakis* sensitization, mainly chronic urticaria, has been frequently reported (Daschner et al., 2013). Currently, it still remains unclear if dead larvae, from previously frozen or adequately cooked fish, can trigger itself an allergenic response (Audicana et al., 2002; Alonso-Gómez et al., 2004; Daschner et al., 2012). To date, there are as far as fourteen recognized larval allergens (Mattiucci et al., 2017), with at least seven of them being excretory/secretory products (D'Amelio et al., 2020).

In recent years, allergic anisakiosis cases have increased, with an estimated global prevalence of 23.1% and the highest rates in Portugal, Norway, and Spain (Rahmati et al., 2020). *A. simplex* s.s. is considered to be the most implicated species in allergic anisakiosis (Ivanović et al., 2017), but also *A. pegreffii* is known to cause allergic reactions, for example in Italy (Mattiucci et al., 2013). In this country, marinated anchovies are the most common fish source of allergic anisakiosis (AAITO-IFIACI *Anisakis* consortium, 2011).

The diagnosis is mainly based on clinical manifestations together with patient history reporting the consumption of raw or undercooked fish. However, unspecific symptomatology can lead to misdiagnosis (Shimamura et al., 2016). Upper endoscopy is the definitive method to diagnose gastric anisakiosis (Bucci et al., 2013). Intestinal anisakiosis is more frequently misdiagnosed as appendicitis, peritonitis, or intestinal obstructions. Also, the difficulty of using upper endoscopy may lead to the need for exploratory laparotomy (Takei and Powell, 2007). Serological assays can be useful especially for allergic anisakiosis, although antigenic cross-reactivity can hamper the diagnosis (Ubeira, 2014).

Treatment is based on the removal of the alive larvae, either by upper endoscopy, colonoscopy, or during surgical intervention (Audicana et al., 2007). Drug therapy based on antihelmintics is not effective (Shimamura et al., 2016).

2.3.4. Prevention and control

Different strategies must be combined to avoid or reduce the risk of ingesting alive larvae of *Anisakis* through the consumption of raw or undercooked fish products.

Epidemiological studies are a key step in assessing health risks in foodborne parasitic diseases. For anisakiosis, the cosmopolitan distribution of the nematode and its complex life cycle involving several hosts, together with international commercial trade of fishery products, hampers its prevention. However, anisakiosis risk can be considered negligible for farmed-raised species, as the life cycle cannot

be completed because of feeding with extruded feed and net pens (Fioravanti et al., 2021; González et al., 2020).

The easiest but most effective strategy is to instruct people on the need to avoid the consumption of fish or fish products that have not been adequately cooked or frozen. *Anisakis* larvae inactivation occurs when cooking fish at 60 ºC for at least one minute or by freezing at -20 ºC for 24 h (González et al., 2020). These prevention measures for the general population have been translated into recommendations made by public health agencies and experts for specific sectors (e.g., the seafood industry), and finally, on legislation. In the European Union, freezing (≤ -20 ºC, for at least 24 h) is mandatory for fishery products in the following cases: (1) those products intended to be eaten raw or undercooked; (2) fishery products of some species, namely herring, mackerel, sprat, and Atlantic and Pacific wild salmon, when cold smoking (< 60 ºC) is the only treatment; and, (3) pickled or salted fishery products (Regulation (EC) No 853/2004).

2.3.5. Current state of research and future directions

There are some aspects of *Anisakis* that have been widely studied, like taxonomy and phylogeny or epidemiological aspects. However, there are other several aspects for which the existing knowledge is still scarce, hampering biomedical research toward effective diagnosis and treatment. To date, the absence of a highly sensitive, specific, and easy-applying diagnostic technique and the unawareness of the professional medical staff in some regions have led to the infra-diagnosis of anisakiosis (Herrador et al., 2019; Seal et al., 2020). Additionally, the lack of drug targets explains the absence of effective medical treatment, now relying more on prevention (EFSA, 2010). Moreover, the comprehension of the pathogenic mechanisms, including a potential tumorigenic effect (Eskesen et al., 2001; Mineta et al.; 2006), as well as the differential pathogenic potential between species, is still in its infancy (Cavallero et al., 2022a).

In recent years, advances in high-throughput sequencing as well as bioinformatics,

in combination with *in vitro* and *in vivo* studies, have pushed a variety of –omic studies resulting in increased knowledge of different biological aspects of *Anisakis* (host-parasite interaction, physiopathological pathways, pathogenic mechanisms or allergen identification and characterization). This basic research is still developing and has the potential to be translated into applied biomedical research focused on the diagnosis, treatment, and prevention of anisakiosis. For example, identifying potential new drug targets or identifying new molecules that can serve as infection markers (D'Amelio et al., 2020; Cavallero et al., 2022a).

Alive *Anisakis* nematodes, mostly infective L3, isolated from naturally infected fish have been the main biological material used in the different studies. However, relying on environmental sources can be limiting because of their availability and the developmental stage needed (Mladineo et al., 2023). The setting up of a reliable protocol for *in vitro* culture of zoonotic nematodes is an efficient tool for providing higher numbers of different developmental stages, more homogenous populations, and/or enabling long-term survival of experimental specimens, which are necessary conditions for some protocols (Knox et al., 2007; Cheong et al., 2021). Therefore, sometimes research must focus on filling gaps in basic knowledge, for example, parasites *in vitro* life cycles, to better pursue translational research.

3. Marine fish as carriers of emerging waterborne unicellular parasites

3.1. Emerging waterborne unicellular parasites

Waterborne diseases occur worldwide due to the contamination of drinking water or by the accidental ingestion of contaminated bathing or recreational waters, including natural water bodies and pools (Karanis et al., 2007). Among parasites, protozoa are the most important group. They are found contaminating several water sources worldwide, increasing the risk of waterborne protozoan outbreaks.

Although most of the recorded outbreaks are caused by *Cryptosporidium* and *Giardia*,

many other protozoa, including amoebas, ciliates, and other sporozoans, are transmitted through contaminated water and have been identified as causative agents of waterborne outbreaks (Plutzer and Karanis, 2016) (**Figure 8**). Microsporidia are generally included in this group of waterborne protozoa. However, their taxonomical classification has been controversial for decades and they have been finally recognized as fungi (Adl et al., 2019). Similarly occurs with *Blastocystis* sp. This parasite belongs to the Heterokonta -or Stramenopiles- phylum, one of the major divisions within the Eukaryota. Traditionally, Heterokonta members have been considered within the protist kingdom, but some current classifications, mainly *Catalogue of Life*, consider them into a recent new kingdom, namely Chromista (Ruggiero et al., 2015). Therefore, we prefer to refer herein as waterborne unicellular parasites, instead of waterborne protozoa. More precisely, as emerging waterborne unicellular parasites (EWUP), as most of the aforementioned parasites are considered to be emergent (NIAID, 2018).

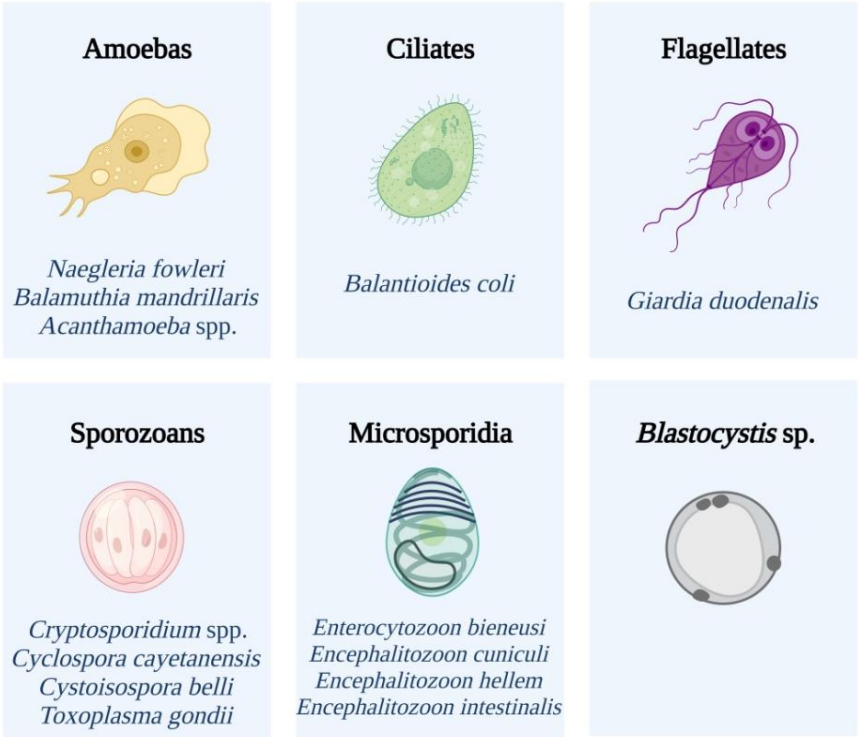

Figure 8. Main emerging waterborne unicellular parasites (EWUP). Created with BioRender.com.

Four major reviews have revised and compiled the overall of waterborne outbreaks registered worldwide since the beginning of the last century, which can be attributed to EWUP (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2017; Ma et al., 2022). It is worth noting that the number of outbreaks has significantly increased over the years. Karanis et al., (2007) reported 325 outbreaks during a period encompassing almost a century. The first register was during the First World War, then a leap until the late 40s and 50s, and, finally, most of the cases were recorded from the last decades of the century. Baldursson and Karanis (2011) found as far as 199 outbreaks reports caused by EWUP during the short time span between 2004 and 2010; Efstratiou et al. (2017) recorded 381 outbreaks in an even shorter period (2011-2016); finally, Ma et al. (2022) registered 251 outbreaks from 2017 to 2020 (although they included outbreaks occurred before 2017 but later published, and therefore not included in the previous reviews).

Waterborne outbreaks are linked to sanitary deficiencies, mainly lack of water sanitization, and therefore more likely to affect poor regions. Also, the impact of these waterborne diseases is of greater magnitude in developing countries (Yongsi, 2010; Kotloff et al., 2013; Siwila et al., 2020). However, the vast majority of EWUP outbreaks are reported in developed regions; America (especially the USA) and Europe (mainly the UK and Ireland), have registered the high number of outbreaks in the most recently assessed years, although Australia was leading the ranking during some previous years (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2017; Ma et al., 2022). This does not necessarily reflect the reality and it is more likely due to the surveillance programs and agencies existing only in developed countries (Efstratiou et al., 2017), together with the availability of appropriate and sensitive diagnostic methods (Adeyemo et al., 2018).

Over the years, most of the EWUP outbreaks have been attributed to *Cryptosporidium* spp., followed by *Giardia duodenalis*. During the 2017-2020 period, they accounted for 76.49% and 19.12% of the total outbreaks, respectively (Ma et al., 2022). The remaining percentage is shared among all other EWUP. *Cryptosporidium*

and *Giardia* are well-studied parasites that are easily transmissible due to their monoxenous life cycles, their zoonotic potential, and the high environmental resistance of their infective forms, being even resistant to industrial water treatments like chlorination (Karanis et al., 2007; Samarro Silva and Sabogal-Paz, 2021). These facts could contribute to explaining their presence and role as waterborne pathogens in developed countries and not only in poor regions where raw and untreated water is used for drinking, cooking, and bathing (Yongsi, 2010; Siwila et al., 2020).

Several other EWUP share these characteristics, but they have received little attention and seem to be underrepresented. Because of that, some authors consider them neglected (Plutzer and Karanis, 2016) and argue that inattention and the lack of rapid and sensitive diagnostic techniques to recover and detect the environmental forms of these parasites could be responsible for their underreporting (WHO, 2004). Fortunately, during the last few years, some improvements have been made in the detection of some of these neglected EWUP (Plutzer and Karanis, 2016), so reports should start to be more representative in the near future.

3.2. Presence in marine environment

Natural body waters can be also contaminated with EWUP through different sources like urban, suburban, and agricultural surface run-off or sewage contaminated by infected humans and/or animals, both domestic (pets and livestock) and wildlife. Not only freshwater bodies are affected, but also the coastal and marine environment, where some of these parasites are known to parasitize and cause disease in different marine mammal species. Moreover, several shellfish are recognized to filtrate and accumulate some of these EWUP, posing a risk to human health (Fayer et al., 2004). There is also a potential risk from recreational bathing in coastal environments, even more, if we take into account that it has been demonstrated that the (oo)cysts of some of the main EWUP (*Cryptosporidium*, *Giardia*, and *T. gondii*) are able to resist the salinity (Brown et al., 1999; Nasser et al.,

2003; Lindsay and Dubey, 2009). However, to date, among the outbreaks associated with recreational waters, none of them have been reported as having the origin in a seawater source (i.e., coastal, estuarine, or marine aquatic environments).

Although reports on EWUP presence in marine waters are scarce, some examples are found in the literature. *Cryptosporidium* and, to a lesser extent, *Giardia*, have been reported from different coastal environments (Robertson, 2007; Graczyk et al., 2010; Cristiane Pinto et al., 2020; Vieira et al., 2022). Some recent reports exist also for *Blastocystis* (Koloren et al., 2018; Vieira et al., 2022), the zoonotic microsporidian *Enterocytozoon bieneusi* (Graczyk et al., 2010), or some amoebas, namely *Entamoeba* (Vieira et al., 2022) and the free living *Acanthamoeba* spp. (Latifi et al., 2020; Salahuldeen et al., 2020).

Conversely, there are a large number of studies on the presence of EWUP in marine shellfish, highlighting the important role that shellfish play as centinels in monitoring marine environment contamination (Lucy et al., 2008). Most of the reports are from *Cryptosporidium*, *Giardia*, and *T. gondii* (Moratal et al., 2020), but other studies reported also the presence in molluscan shellfish of *C. cayetanensis* (Aksoy et al., 2014), microsporidia (Lucy et al., 2008), and *Blastocystis* (Martínez-Barbabosa et al., 2017).

3.3. Potential role of marine fish in transmission

In recent years, this knowledge on EWUP entering marine ecosystems has enhanced the question about the possibility of other marine organisms, mainly fish, acting either as hosts or mechanical carriers for these parasites of terrestrial origin. Similar to the way in which molluscan shellfish filter the seawater and retain the parasitic forms, fish can get them during feeding, then reach the digestive system, or during breathing, being retained in gill filaments.

The role of fish as potential actors in EWUP life cycles has been studied only for *Cryptosporidium* spp., *G. duodenalis*, and *T. gondii*, relying on freshwater experimental

models (review in Moratal et al., 2020). During the 90s, experimental infections were performed to ascertain if fish (aquarium fish and rainbow trout) could be truly infected by *Cryptosporidium parvum* (Arcay et al., 1995; Graczyk et al., 1996; Freire-Santos et al., 1998). These studies showed contradictory and not conclusive results, but they presented several limitations, such as the absence of molecular characterization, the use of different host species, and the employment of different analytical methods. An attempt at *G. duodenalis* experimental infection in zebrafish (*Danio rerio)* was also conducted and seemed to indicate that a true infection cannot be achieved (Tysnes et al., 2012). Finally, experimental infections for *T. gondii* were also performed. Omata et al. (2005) did not succeed in establishing the infection in the goldfish (*Carassius auratus*), while Sanders et al. (2015) were able to establish a successful acute infection in zebrafish. Akin to *Cryptosporidium*, the results were contradictory, but these studies used different species, different inoculation sites, and different methods of analysis. However, for *T. gondii* there are other evidences that suggest primary infections in fish (Taghadosi et al., 2010; Yoshida et al., 2020).

The question of whether fish could be infected by these parasites remained unclear. However, even if fish are acting only as mechanical carriers, they can still be a source of infection for humans, through seafood consumption, like shellfish are (Moratal et al., 2020). Therefore, epidemiological studies on cultivated and wild fish aiming to detect the presence of these parasites are of high importance for public health.

Some molecular epidemiological surveys have been conducted to detect the presence of zoonotic *Cryptosporidium* species, *G. duodenalis*, *T. gondii*, and *Blastocystis* sp. in fish (Gantois et al., 2020; Moratal et al., 2020; Rauff-Adedotun et al., 2022). However, information is still scarce, specifically concerning marine fish (**Table 1**).

Table 1. Studies performed on marine fish targeting emerging waterborne unicellular parasites (EWUP).

Parasite	Location	Zoonotic species/variants identified	Study
***Blastocystis* sp.**	Boulougne sur-Mer (Atlantic Northeast zone 27)	Subtypes ST2, ST7, ST8	Gantois et al., 2020
***Cryptosporidium* spp.**	Western Australia (Indian ocean zone 57.5)	*C. parvum*, *C. xiaoi*, pig genotype II	Reid et al., 2010
	Papua New Guinea (Pacific Western Central zone 71)	*C. parvum*, *C. hominis*	Koinari et al., 2013
	European seas	*C. parvum*	Certad et al., 2019
Giardia duodenalis	Western Australia (Indian ocean zone 57.5)	Genotypes A (A2) and B (B3, B4)	Yang et al., 2010
Toxoplasma gondii	Sicily (Central Mediterranen zone 37.2.2)	*Toxoplasma gondii*	Marino et al., 2019

JUSTIFICATION AND OBJECTIVES

Despite the beneficial nutritional impact of marine fish on human health, different risks arise from its consumption, such as fishborne infectious diseases, including parasitic ones. For the purpose of this thesis we have made a distinction between zoonotic marine fish true parasites, i.e., parasites for which marine fish are natural hosts, like *Anisakis* spp.; and zoonotic parasites with a terrestrial origin that fish can harbor, without being necessarily infected (referred in the introduction as EWUP). As overviewed in the introduction, the state of knowledge is quite different between both groups, presenting different research needs. For this reason, this thesis encompasses two differentiated parts, as follows.

Information on the presence of EWUP in marine fish is still scarce. However, epidemiological information on their geographical and host distribution, relying on molecular identification, is the first essential step to determining whether or not a risk of acquiring these parasitic diseases through fish consumption exists. This is particularly relevant in regions with high fish consumption, such as Mediterranean countries, and Spain in particular. Therefore, the first main and specific objectives of this thesis were:

1. To detect and genetically identify unicellular (re)emerging parasites with zoonotic potential in edible marine fish from the western Mediterranean, Spanish coast:
    1.1. To study the presence and genetically identify *Cryptosporidium* spp. in farmed and wild fish from the Comunidad Valenciana, Spain.
    1.2. To study the presence and genetically identify *Blastocystis* sp. in farmed and wild fish from the Comunidad Valenciana, Spain.
    1.3. To study the presence and genetically identify zoonotic microsporidia species in farmed and wild fish from the Comunidad Valenciana, Spain.
    1.4. To assess the parasite community, including zoonotic unicellular parasites, in the gastrointestinal tract of the European pilchard (*Sardina pilchardus*) and sympatric round sardinella (*Sardinella aurita*) using a dietary metabarcoding

approach.

On the other site, we found marine fish true parasites that are well-known causes of fishborne parasitic infections, like flukes or nematodes. The second part of this thesis focuses on *Anisakis pegreffii*, because of its public health significance and status as the predominant *Anisakis* species in the Mediterranean. Current research on *Anisakis* spp. is more focused on host-parasite interaction, pathogenesis, diagnosis, and treatment. The development of an *in vitro* life cycle protocol can facilitate the understanding of developmental and physiological features with relevance in the host-parasite interaction and provide biological material for -omic studies. For this reason, the second main and specific objectives of this thesis were:

2. To contribute to the *in vitro* life cycle of the emerging zoonotic nematode *A. pegreffii*.
   2.1. To determine the *in vitro* fecundity of *A. pegreffii*.
   2.2. To optimize the protocol for culturing the second-stage larva (L2) of *A. pegreffii* and to describe key ultrastructural features of early larval development.
   2.3. To obtain the karyotype of *A. pegreffii*.

CHAPTER 1

CHAPTER 1. Occurrence and molecular identification of unicellular emerging zoonotic parasites in cultivated and wild marine fishes from western Mediterranean: *Cryptosporidium* spp., *Blastocystis* sp., and Microsporidia

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The content from this chapter encompasses the studies published in the following articles, together with unpublished information on *Blastocystis* sp.:

1. Moratal, S., Dea-Ayuela, M. A., Martí-Marco, A., Puigcercós, S., Marco-Hirs, N. M., Doménech, C., et al., 2022. Molecular characterization of *Cryptosporidium* spp. in cultivated and wild marine fishes from Western Mediterranean with the first detection of zoonotic *Cryptosporidium ubiquitum*. Animals, 12(9), 1052. https://doi.org/10.3390/ani12091052
2. Moratal, S., Magnet, A., Izquierdo, F., del Águila, C., López-Ramon, J., Dea-Ayuela, M.A., 2023. Microsporidia in commercially harvested marine fish: a potential health risk for consumers. Animals, 13(16), 2673. https://doi.org/10.3390/ani13162673

1. Introduction

Cryptosporidium (Phylum Apicomplexa) is a widespread intracellular protozoan parasite with a complex direct life cycle. The oocyst constitutes the infective form, which exhibits high environmental resistance. Following the ingestion of the infective oocyst by the host, the excystation takes place in the small intestine releasing the sporozoites that parasitize the host enterocytes, leading to the formation of a parasitophorous vacuole. Within this vacuole, the parasite undergoes mitotic division, yielding multiple merozoites. These merozoites escape and infect other cells, repeating the asexual cycle. Alternatively, merozoite infection may result in the formation of a macrogamont (female) or a microgamont containing several microgametes (male). Fertilization leads to the development of a diploid zygote, which differentiates into an oocyst. Meiosis within the oocyst generates four sporozoites. The sexual cycle concludes with two possible outcomes: a thin-walled oocyst (20%) that excysts in the host for autoinfection or a thick-walled oocyst (80%) that is excreted into the environment (Leitch and He, 2011).

Cryptosporidium comprises several species that infect a wide range of wild and domestic vertebrates (Santín, 2013; Zahedi et al., 2016). The most prevalent species in humans are anthroponotic *Cryptosporidium hominis* and zoonotic *Cryptosporidium parvum*, although there are many other zoonotic species such as *Cryptosporidium meleagridis* or *Cryptosporidium canis* (Ryan et al., 2014). It is considered an important water- and foodborne pathogen all over the world. Indeed, it is documented as the fifth most important foodborne pathogen, with more than eight million cases reported annually (FAO/WHO, 2014); and it is also the main protozoa involved in waterborne outbreaks (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2017; Ma et al., 2022). It is a major cause of diarrhea worldwide, especially in young children (Levine et al., 2020), and may even be a life-threatening pathogen in immunocompromised people (Chalmers and Davies, 2010).

Concerning fishes, *Cryptosporidium* has been detected in several hosts, including cultivated and wild freshwater and marine species, as well as ornamental fishes. *Cryptosporidium* spp. characterization in fish is based mainly on morphological description and, chiefly, on molecular methods, which are essential to identify species, genotypes, and subgenotypes (Xiao, 2010). Currently, five species are recognized as specific to fish hosts (**Table 2**). Several genotypes specific to fish (piscine genotypes) have been documented, too (reviewed in Golomazou et al., 2021).

Table 2. Fish-specific *Cryptosporidium* species.

Species	Host type	References
C. molnari	Gilthead seabream (*Sparus aurata*) European seabass (*Dicentrarchus labrax*)	Alvarez-Pellitero and Sitjà-Bobadilla, 2002 Palenzuela et al., 2010
C. scophthalmi	Turbot (*Scophthalmus maximus*)	Costa and Saraiva, 2015
C. huwii	Guppy (*Poecilia reticulata*) Golden tiger barb (*Puntigrus tetrazona*) Neon tetra (*Paracheidon innesi*)	Ryan et al., 2015 Yang et al., 2015 Paparini et al., 2017
C. bollandi	Oscar fish (*Astronotus ocellatus*)	Bolland et al., 2020
***C. abrahamseni* n. sp.**	Red-eye tetras (*Moenkhausia sanctaefilomena*	Zahedi et al., 2021

Additionally, non-piscine host-specific species and genotypes have been detected in fish. The high environmental oocyst resistance (Samarro Silva and Sabogal-Paz, 2021) allows the contamination of aquatic environments with oocysts coming from terrestrial species (generally by fecally contaminated wastewater or agricultural run-off), entering even coastal environments (Graczyk et al., 2010; Cristiane Pinto et al., 2020; Vieira et al., 2022) and accumulating in marine organisms like shellfish and fish (Moratal et al., 2020). Among these terrestrial species, zoonotic *Cryptosporidium*

parvum, anthroponotic *Cryptosporidium hominis*, *Cryptosporidium xiaoi*, *Cryptosporidium scrofarum*, and rat genotype III have been detected in fishes (Golomazou et al., 2021).

Blastocystis sp. (Phylum Stramenopiles) is an anaerobic and polymorphic protist found worldwide. Depending on the classification, it may also be categorized within the kingdom Chromista (Ruggiero et al., 2015). Its biological life cycle is still not fully understood, but at least six stages have been identified from cultures and faeces: vacuolar, granular, ameboid, multivacuolar, avacuolar, and cyst. The cyst is believed to be the infectious form transmitted through the fecal-oral route. In the simplest case, excystation takes place in the large intestine, resulting in the vacuolar form. The vacuolar form divides primarily through binary fission and can give rise to either the ameboid or the granular forms. Encystation results in the formation of the infectious cyst, which is expelled through faeces (Parija and Jeremiah, 2013).

Blastocystis sp. is present in humans and in a wide range of animals, including mammals, birds, reptiles, amphibians, and insects (Rauff-Adedotun et al., 2021). Currently, it is considered the most frequent enteric micro-eukaryote detected in humans, both in developed and developing countries, although with higher prevalences in the latter (Lokmer et al., 2019). *Blastocystis* sp. shows high genetic variability according to the phylogeny of the 18S ribosomal ribonucleic acid (18S rRNA) gene, with at least 34 subtypes (STs) described and validated to date: ST1-ST17, ST21, and ST23-38 (Tantrawatpan et al., 2023). Humans are affected by at least ten of them (ST1-ST9, and ST12), the most predominant the ST1-ST4 (Popruk et al., 2021). The others are known to colonize mammals or birds (ST5 in pigs; ST6 and ST7 in birds), which are the potential reservoirs for zoonotic transmission (Wang et al., 2014; Greige et al., 2018). Despite its high presence in humans, it is considered a cryptic parasite. Several aspects of its biology and transmission still remain unclear, including its pathogenicity. While *Blastocystis* sp. is frequently found in asymptomatic individuals (Stensvold and Clark, 2016), it has also been related to unspecific gastrointestinal symptomatology and has been linked to irritable bowel

syndrome and chronic urticaria (Poirier et al., 2012; Bahrami et al., 2020). Different STs are related to different virulence and pathogenicity (e.g., Aykur et al., 2022; Deng et al., 2023).

Although it is recognized as a waterborne parasite this epidemiological aspect is still poor-understood, with only four outbreaks reported worldwide (Karanis et al., 2007; Baldursson and Karanis, 2011; Ma et al., 2022). Attah et al. (2022) reviewed *Blastocytis* sp. presence in water during the last two decades (2005-2022). They found 25 studies reporting its presence in different water sources, with only two reports identifying ST1 and ST3 in seawater from Turkey (Koloren et al., 2018) and Poland (Adamska, 2020). Albeit scarce, this information indicates that *Blastocystis* sp. is able to enter marine ecosystems. The presence of *Blastocystis* sp. in marine ecosystems, together with the high genetic and host variability and the potential for zoonotic transmission, poses the question of the existence of this unicellular parasite in marine organisms. To our knowledge, there is a single recent study reporting for the first time the presence of different *Blastocystis* sp. STs in marine fish and marine mammals from the Northeast Atlantic (Gantois et al., 2020).

Microsporidia is a wide and diverse phylum that comprises more than 1,500 species of obligate intracellular and spore-forming parasites, currently classified as fungi (Adl et al., 2019). The general life cycle comprises three phases. During the infective or environmental phase, the spores are liberated and dispersed in the environment, being environmental conditions a key factor for its germination; spores constitute the free-living, resistant, and infective form. Hosts could become infected by ingestion or inhalation of the spores. Once in the host, the spores infect the cells actively by either injecting their sporoplasm through a unique structure, the extrusion apparatus, or passively by being phagocytized (Cali and Takvorian, 2014). Recent studies suggest a third via of infection by the formation of a synapsis pocket at the surface of the host cell mediated by the polar tube proteins, where the sporoplasm is deposited and lately engulfed inside the host cell (Han et al., 2022). Independently of how microsporidia infect the host cell, the proliferative and

sporogonic phases occur inside the cell. Depending on the species, this could occur in the cytoplasm of the host-infected cell, inside a parasitophorous vacuole, or inside the nucleus (Cali and Takvorian, 2014).

Microsporidia infects an extensive host range, comprising both vertebrates, including humans, and invertebrate hosts. Almost half of the 220 microsporidia genera described are known to infect aquatic organisms (Cali et al., 2017), with about 20 genera infecting fish (Stentiford et al., 2013). According to the microsporidia species and tissue affected, different clinical and pathological signs can be observed in fish, like emaciation, growth inhibition, or leukemia-like syndrome. Moreover, some genera are known to induce xenome formation (*Glugea* spp. or *Loma* spp.) with high tissue destruction (Lom and Dyková, 2005). Consequently, fish microsporidia impact the production of cultivated populations, with significant economic losses in the aquaculture sector. Their ability to infect different tissues and organs leads to reduced growth rates, decreased feed conversion efficiency, and high mortality rates. For example, *Enterospora nucleophila* has been identified as the cause of an emaciative syndrome and mortalities in gilthead seabream (*Sparus aurata*) cultures from Mediterranean waters (Palenzuela et al., 2014). Microsporidian infections can also impact wild fish populations and have been associated with the collapse of some fisheries therefore affecting commercial fishing (Monaghan et al., 2009).

However, little is known about the presence of zoonotic microsporidia in fishes. Currently, nine genera are recognized to cause infection in humans, namely *Anncaliia*, *Encephalitozoon*, *Enterocytozoon*, *Microsporidium*, *Nosema*, *Pleistophora*, *Trachipleistophora*, *Tubulinosema*, and *Vittaforma* (Han and Weiss, 2017). To our knowledge, only the genus *Pleistophora* from poikilothermic hosts (fish and reptiles) has been related to myositis in immunocompromised people; although later analysis classified it in a new species, *Pleistophora ronneafiei*, specific from humans (Mathis et al., 2005). Within these genera, 17 species are known to infect humans (Han and Weiss, 2017). From those, *Enterocytozoon bieneusi* is the most frequently diagnosed

human species, followed by *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi* (Qiu et al., 2019). Microsporidiosis in humans is mainly associated with a diarrheal syndrome and other affections like encephalitis, keratoconjunctivitis, hepatitis, sinusitis, myositis, or disseminated infection, mainly in immunocompromised people (Weiss, 2014; Stentiford et al., 2016). Although in recent years, they are increasingly recognized in immunocompetent populations, suggesting an underreported incidence in healthy populations (Lores et al., 2002; Abreu-Acosta et al., 2005).

Infective spores are very adaptable and resistant to different environmental conditions of temperature, humidity, desiccation, and even water treatments (e.g., water chlorination), facilitating its persistence in the environment and, therefore, its transmission. They are mainly transmitted by the ingestion of contaminated food or water, being the water crucial in the epidemiology of microsporidia species (Weiss, 2014; Stentiford et al., 2016). Human pathogenic microsporidia have been found in different water sources like wastewater, drinking water, or recreational river waters (Dowd et al., 2003; Graczyk et al., 2007; Izquierdo et al., 2011; Galván et al., 2013). Moreover, microsporidia zoonotic species can also reach the marine environment. For example, *E. bieneusi* has been found in recreational beach water from Chesapeake Bay, Maryland (Graczyk et al., 2010), and *E. bieneusi* with *E. cuniculi* have been detected in biofilms from Pensacola Bay, Florida (Moss and Snyder, 2019). Like other waterborne pathogens, zoonotic microsporidia are also detected in shellfish in the river and coastal waters (Graczyk et al., 2004; Lucy et al., 2008). Their presence on shellfish is interesting for biomonitoring purposes but also highlights a potential health risk from aquatic food consumption. Due to their presence in coastal waters and shellfish, we hypothesize that these major zoonotic microsporidia species could also be present in edible marine fishes.

It has not been confirmed that any of these pathogenic parasitic species are actually infecting fish hosts, but their mere presence in edible fish as mechanical carriers would imply, at any rate, a potential risk to public health. Therefore, this study aims

to assess the presence of zoonotic species/subtypes of *Cryptosporidium* spp., *Blastocystis* sp., and Microsporidea in both cultivated and wild fish from the Comunidad Valenciana (Spain). The Comunidad Valenciana is the largest marine aquaculture producer in Spain, generating as far as 16,353 tonnes in 2020 (APROMAR, 2021). This activity coexists with extractive fishing activity, therefore providing an interesting region of study.

2. Material and methods

2.1. Study design and study area

The present survey was conducted in the Comunidad Valenciana, a region located in eastern Spain, whose marine area belongs to FAO zone 37.1.1 (western Mediterranean, Balearic division). This survey included three different fish groups: cultivated fish from off-shore aquaculture farms, wild fish captured in the surroundings of the aquaculture facilities (hereafter, synanthropic fish), and wild fish from commercial extractive fisheries. Cultivated fish were obtained at four fattening off-shore farms belonging to the Agrupación de Defensa Sanitaria de Acuicultura de la Comunidad Valenciana (ADS ACUIVAL) (**Figure 9**). These farms, located at a mean coastal distance of 1.87 nautical miles and at an average depth of 35.75 m, are dedicated to the fattening of three commercially important species, European seabass (*Dicentrarchus labrax*), gilthead seabream, and meagre (*Argyrosomus regius*). The Comunidad Valenciana is the leading producer in Spain for these species, which are among the top ten species produced by aquaculture in the European Union (APROMAR, 2021). This region is characterized by favorable environmental conditions for the growth and development of these species, with suitable water temperatures (ranging from 12 ºC in winter to 28 ºC in summer) and smooth and regular currents ensuring adequate water flow and quality. In this type of production, fish are reared in floating pens with nets of different mesh sizes, therefore allowing the free circulation of water and constituting the only physical barrier with the synanthropic fish population that aggregates around (**Figure 10**).

Fish from extractive fisheries, mainly bottom trawling, came from four different fish markets distributed along the Comunidad Valenciana coast and were randomly chosen from different ships and species to avoid economic losses to fishermen (**Figure 9**).

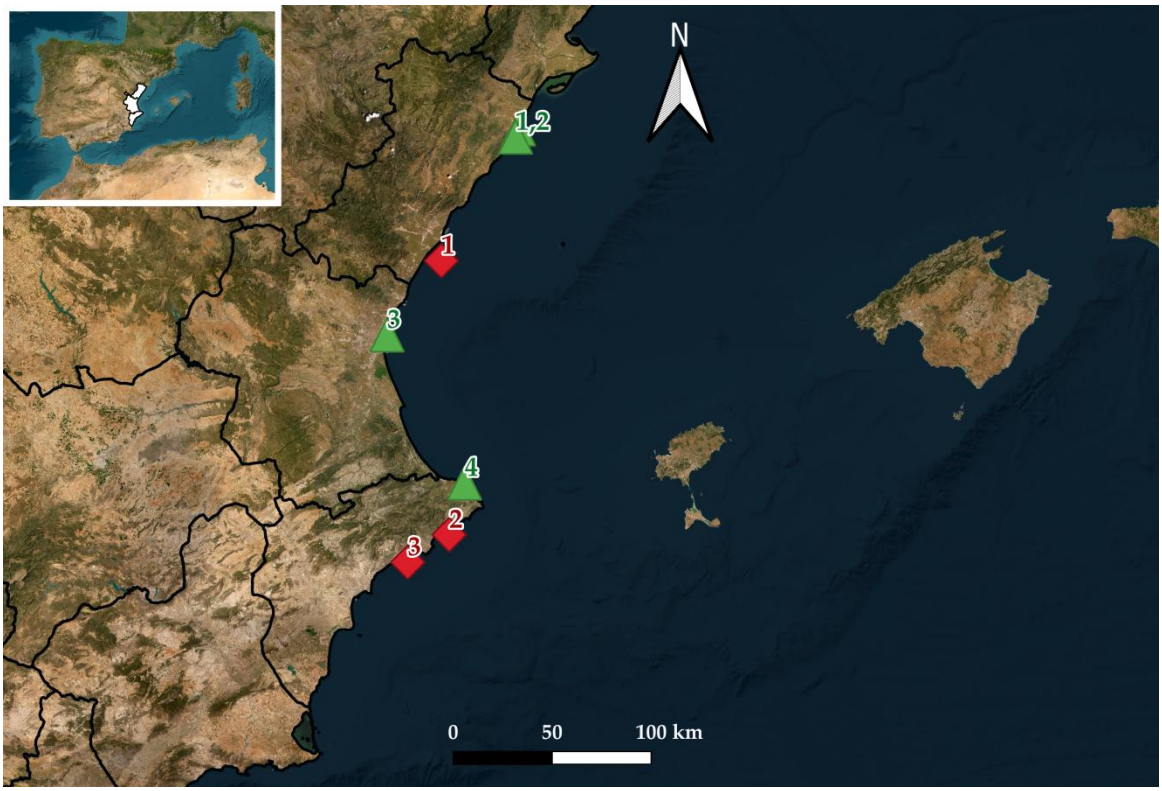

Figure 9. Sampling point locations: farms (red diamonds) and fish markets (green triangles). The fourth farm is not shown for confidentiality reasons.

2.2. Fish sampling

Sampling took place from July 2020 to October 2021, mainly in the autumn and summer seasons, except for the third group (wild fish from extractive fisheries), which was sampled during the second year of the study, from March to June 2021. A total of 408 fishes were sampled, distributed as follows: 147 cultivated fishes, 147 wild synanthropic fishes, and 114 wild fish from extractive fisheries. Fishes from the first two groups were stunned and slaughtered during regular working operations at farms and following the standard procedure used in Mediterranean marine aquaculture, consisting of immersion into a slurry ice solution (thermal shock). Fish

from the third group were sampled once already dead due to commercial fishing activity. Hence, the project supporting this study was exempt from ethics approval, according to the Ethics Committee of Animal Experimentation of the Universidad Cardenal Herrera-CEU (Alfara del Patriarca, Valencia, Spain).

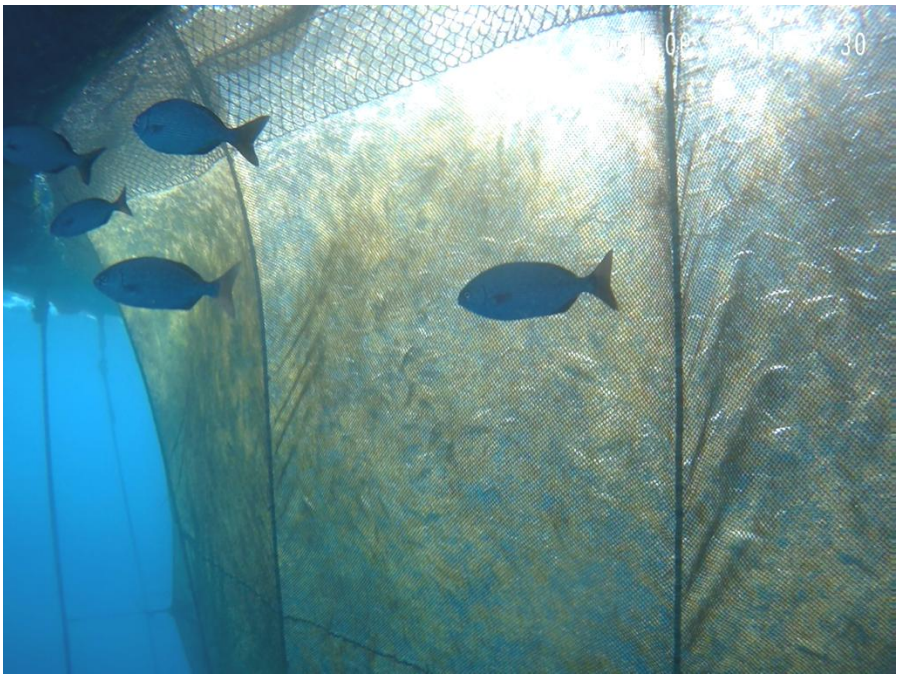

Figure 10. Wild salemas (*Sarpa salpa*) swimming in the surroundings of a floating pen rearing cultivated fish. Own source (PARAPEZ project).

Sampled fish were stored in refrigeration and transportated to the Parasitology laboratory at the Universidad Cardenal Herrera-CEU, to be processed within the first 24 h after death. Each individual was identified at the species level according to Louisy (2006). Body weight and total body length were registered and dissection was performed using sterile dissection material. A sample was taken consisting of gastrointestinal tissue scraping mixed with intestinal content for each individual. Additionally, somatic muscle from the caudal region was sampled in some specimens. Both samples were frozen at -20 ºC until further processing.

2.3. DNA extraction

Approximately 250 mg of each gastrointestinal sample was used for

deoxyribonucleic acid (DNA) extraction. For this purpose, the NZY Tissue gDNA Isolation Kit (Nzytech genes & enzymes, Lisbon, Portugal) was used, following the manufacturer's protocol and including the specific preparation steps for stool samples.

DNA extraction from muscle was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The manufacturer's protocol was followed, considering samples were incubated with the proteinase k overnight (16-17 h). DNA from muscle was preserved at -20 ºC and only used for Microsporidia testing.

2.4. Molecular detection and characterization

2.4.1. Cryptosporidium spp.

DNA from gastrointestinal samples was tested for *Cryptosporidium* spp. following a nested polymerase chain reaction (nested PCR) protocol to amplify a 578 base pairs (bp) fragment of the 18S rRNA, as described by Ryan et al. (2003). Briefly, 3 µL of DNA sample were used in a total amplification reaction volume of 25 µL, containing 12.5 µL of Supreme NZYTaq II 2x Green Master Mix (Nzytech genes & enzymes, Lisbon, Portugal) and 12.5 pmol of each primer pair (18SicF2/18SicR2 and 18SicF1/18SicR1) (**Table 3**). Both PCR amplification reactions were carried out following the original protocol conditions (Ryan et al., 2003) in a thermal cycler GeneAmp PCR System 2700 (Applied Biosystems). Briefly, a denaturation step at 95 ºC for 5 min, followed by 45 amplification cycles (30 s at 94º C, 30 s at 58º C, and 30 s at 72 ºC), and a final extension at 72 ºC for 10 min. All PCR runs included a positive control, consisting of DNA from *Cryptosporidium ubiquitum* isolated from infected farmed lambs, and a negative control without DNA template.

Subsequently, positive isolates were also amplified at the actin locus. For this purpose, two sets of primers were used: (1) general actin primers for *Cryptosporidium* genus, in a nested PCR (Sulaiman et al., 2002); and (2) actin primers specifically designed for piscine-derived *Cryptosporidium* (Koinari et al., 2013), in a

semi-nested PCR (**Table 3**). Both PCRs were carried out according to the original protocols described in Sulaiman et al. (2002) and Koinari et al. (2013), except that we used the Supreme NZYTaq II 2x Green Master Mix (Nzytech genes & enzymes, Lisbon, Portugal) in the reactions.

For both genes, products of the secondary reactions were visualized on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Seongnam, Republic of Korea).

Table 3. Primers used for *Cryptosporidium* spp. characterization.

Genes	Primers	Sequences (5′-3′)	References
18S rRNA	18SicF2	GACATATCATTCAAGTTTCTGACC	Ryan et al., 2003
	18SicR2	CTGAAGGAGTAAGGAACAACC	
	18SicF1	CCTATCAGCTTTAGACGGTAGG	
	18SicR1	TCTAAGAATTTCACCTCTGACTG	
Actin	F1	ATGRGWGAAGAAGWARYWCAAGC	Sulaiman et al., 2002
	R1	AGAARCAYTTTCTGTGKACAAT	
	F2	CAAGCWTTRGTTGTTGAYAA	
	R2	TTTCTGTGKACAATWSWTGG	
Actin	ActinaIIF1	GTAAATATACAGGCAGTT	Koinari et al., 2013
	ActinaIIR1	GGTTGGAACAATGCTTC	
	ActinaIIF2	CCTCATGCTATAATGAG	

2.4.2. Blastocystis sp.

For *Blastocystis* sp. detection in gastrointestinal samples, *Blastocystis*-specific primers BL18SPPF1 (5′-AGTAGTCATACGCTCGTCTCAAA-3′) and BL18SR2PP (5′-TCTTCGTTACCCGTTACTGC-3′) targeting a 320-342 bp fragment of the 18S rRNA gene (Poirier et al., 2011) were used in an end-point PCR protocol, adapted from Gantois et al. (2020). The reaction was performed by adding 2 µL of DNA and 0.5 µM of each primer in a 50 µL total reaction volume and using the same Master Mix as above, Supreme NZYTaq II 2x Green Master Mix (Nzytech genes & enzymes,

Lisbon, Portugal). Amplification was performed in thermal cycler GeneAmp PCR System 2700 (Applied Biosystems), using cycling conditions described in Gantois et al. (2020): a denaturation step at 95 ºC for 5 min, followed by 40 cycles of amplification (30 s at 94 ºC, 35 s at 60 ºC, 50 s at 68 ºC), and 2 min at 68 ºC for final extension. Each PCR run included a negative control without genomic DNA template and a positive control isolated from an infected piglet (*Blastocystis* sp. ST5). Final products were visualized in 1.8% agarose gel stained with GreenSafe Premium (Nzytech genes & enzymes, Lisbon, Portugal).

2.4.2.1. Next generation amplicon sequencing and bioinformatic analysis

With the purpose of identifying potential mixed infections with different STs, next generation amplicon sequencing was applied to positive samples. The analysis was performed by Sequencing Multiplex S.L. (Paterna, Valencia, Spain). We applied the protocol according to Maloney et al. (2019), with minor modifications. PCR was performed using primers Blast505_532F and Blast998_1017R described by Santín et al. (2011), but incorporating Illumina adapter sequences: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGGTAGTGACAATAAATC-3'; 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTTTCGCACTTGTTCATC-3'. The library preparation was performed using the Illumina Metagenomic Sequencing Library Preparation protocol (Part# 15044223 Rev. B), including a dual indexing strategy. Cycling conditions for the amplicon PCR step were as follows: 95 ºC for 5 min, 35 cycles of 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, and a final elongation consisting of 72 ºC for 5 min. Subsequently, samples were analyzed using the QIAxcel Advanced System (Qiagen, CA, USA), and obtained libraries were quantified using Qubit Fluorometric Quantitation assay (Thermo Fisher Scientific, MA, USA). A final pooled library concentration of 5 pM with 20% PhiX control was sequenced on an Illumina MiSeq using 500 cycle v2 chemistry (250 base pair, paired-end reads) (Illumina, CA, USA).

Due to the low performance shown by Illumina 600 cycle v3 chemistry, the 500 cycle v2 chemistry was used. Consequently, the resulting amplicon was slightly shorter

than the original (≈ 510 bp) and reads did not overlap. This was solved during bioinformatic analysis, by intercalating N nucleotides between the two reads during the merging and by modifying the Basic Local Alignment Search Tool (BLAST) algorithm.

After demultiplexation and primers elimination, sequences were subjected to a quality analysis using FastQC software (version 0.11.9). Afterward, paired-end reads were analyzed using software DADA2 software (Callahan et al., 2016). The pipeline includes the following steps: trimming and filtering of the sequences according to standard quality parameters; application of the learnErrors method to correct potential sequencing errors; merging forward and reverse reads; construction of the amplicon sequence variants (ASVs) table to obtain all single full amplicons with their relative frequencies; to remove chimeras. Finally, taxonomy assignation to each ASV was performed by comparison with an ad-hoc *Blastocystis* sp. database (extracted from the National Center for Biotechnology Information, NCBI) and using the BLAST tool (Altschul et al., 1990).

2.4.3. Microsporidia

Human pathogenic microsporidia species detection and identification was conducted through a SYBR Green real-time PCR, described by Polley et al. (2011) and adapted by Andreu-Ballester et al. (2013), targeting a region of the 18S rRNA gene. QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) was used in a total reaction volume of 20 µl containing 5 µl of template DNA. MsRTf1 (5'-CAGGTTGATTCTGCCTGACG-3') and MsRTr1 (5'-CCATCTCTCAGGCTCCCTCT-3') primers were added at 0.5 µM final concentrations. Amplification was performed in an Mx3000 qPCR System (Agilent, Santa Clara, CA, United States) with the following cycling conditions: 50 ºC for 2 min, 95 ºC for 15 min, 40 cycles of 95 ºC for 10 s, 60 ºC for 20 s, and 72 ºC for 20 s. A final melting cycle was set up at 95 ºC for 1 min and 55 ºC for 30 s.

This PCR allows for identifying the most common zoonotic microsporidia species

infecting humans according to specific melting temperatures (Tm). *E. cuniculi* amplifies at 84.45 ± 0.4 ºC, *E. intestinalis/hellem* at 82.85-83.9 ºC (this PCR assay does not permit to distinguish between both species), and finally, *E. bieneusi* at 82.35 ± 0.4 ºC. This PCR test was designed for human diagnosis; therefore, the possible cross-reaction with other microsporidia marine species was not studied. This is particularly relevant in the case of *E. bieneusi* since all other species of this family (Enterocytozoonidae) are typically fish or shellfish parasites. That is why the results are presented as either the absence or presence of Enterocytozoonidae. By contrast, *E. intestinalis/hellem* pertains to a family, even extendable to the whole clade, which is eminently terrestrial, with only one marine species parasite of a free-living nematode (Park and Poulin, 2021), thus eliminating the possibility of cross-reactions.

When available, muscle from fishes that were positive at the gastrointestinal tract for Enterocytozoonidae or *E. intestinalis/hellem* was also analyzed with the same technique to determine if these zoonotic species could reach the edible muscular tissue.

2.5. Sequence and phylogenetic analyses

Positive isolates of *Cryptosporidium* spp. and *Blastocystis* sp. with a band of the expected size were Sanger-sequenced by an external sequencing service (Principe Felipe Research Centre, Valencia, Spain) using an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA). Chromas version 2.6.6 (Technelsyum DNA Sequencing Software, South Brisbane, QLD, Australia) was used to visualize and fit the nucleotide sequences obtained. Fitted sequences were compared with *Cryptosporidium* spp. and *Blastocystis* sp. sequences deposited in the NCBI GenBank database, using the online BLAST tool (Altschul et al., 1990).

For *Cryptosporidium* spp. sequences, phylogenetic analyses were conducted using MEGA version 11 (Tamura et al., 2021). Sequences were aligned to selected reference sequences, pairwise distance matrixes were calculated, and phylogenetic

trees for the 18S rRNA and actin genes loci were constructed by the Maximum Likelihood (ML) method using the Tamure 3-parameter substitution model. Bootstrap tests were conducted on 1000 replicates.

2.6. Data analysis

Fish mean weights and mean total body lengths ± standard deviation (STD) were calculated. Prevalence, expressed as mean ± standard error (SE), was calculated for each parasite (*Cryptosporidium* spp. and *Blastocystis* sp.) and fish group. Microsporidia were expressed in terms of presence (instead of prevalence), since in this case the achieved sampled size was not sufficient to estimate prevalence. Fisher's exact test was applied to assess differences between the groups' prevalences. The significance level was set at a p-value ≤ 0.05. Analyses were performed on R software version 4.1.2 (R Core Team, 2021).

3. Results

3.1. Sampled fish

Three species of cultivated fish were sampled from four offshore fattening farms. From the overall 147 cultivated specimens, 57.1% (N=84) were European seabass, 26.5% (N=39) were gilthead seabream, and 16.3% (N=24) were meagre (**Supplementary table 1**).

Synanthropic wild fish captured in the surroundings of these four farms (N=147) comprised 18 different species, with most of the specimens belonging to only six of them: the Mediterranean horse mackerel (*Trachurus mediterraneus*) (N=31; 21.1%), the axillary seabream (*Pagellus acarne*) (N=25; 17%), the round sardinella (*Sardinella aurita*) (N=25; 17%), the common two-banded seabream (*Diplodus vulgaris*) (N=16; 10.9%), the blotched picarel (*Spicara maena*) (N=11; 7.5%), and the European seabass (N=10; 6.8%) (**Supplementary table 2**).

Finally, wild fish acquired at fish markets (N=114) was a heterogeneous group

composed of 31 different species. However, about half (54.4%) was represented by only five species: the Atlantic mackerel (*Scomber scombrus*) (N=22; 19.3%), the pouting (*Trisopterus luscus*) (N=12; 10.5%), the European hake (*Merluccius merluccius*) (N=11; 9.6%), the red mullet (*Mullus barbatus*) (N=9; 7.9%), and the greater forkbeard (*Phycis blennoides*) (N=8; 7%). Fifteen species corresponded to unique specimens (**Supplementary table 3**).

3.2. *Cryptosporidium* spp.

Cryptosporidium spp. were analyzed on the three sampled fish groups: cultivated fish (N=147), synanthropic fish (N=147), and wild fish from extractive fisheries (N=110).

Cryptosporidium spp. was detected in 17 out of 404 samples (4.2 ± 1%). Prevalence in the cultivated group was 4.8 ± 1.8% (7/147), while prevalence in wild fish was 3.9 ± 1.2% (10/257). Among wild fish, the prevalence was higher in the group of synanthropic fishes (6.1 ± 2%; 9/147), with only one fish infected from extractive fisheries (0.9 ± 0.9%; 1/110). The difference in prevalence between these two groups was statistically significant (p = 0.047). Positive isolates were found in synanthropic fish from the four marine farms studied and in cultivated fish from three of these farms (**Figure 11**).

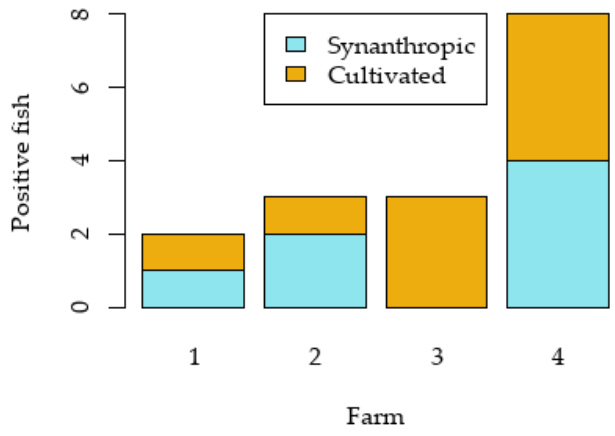

Figure 11. Number of positive fishes according to group and location (only applied to farms).

Cryptosporidium spp. was detected in all of the three cultivated species analyzed, in four European seabass, two gilthead seabream, and one meagre. Regarding synanthropic fishes from farm surroundings, *Cryptosporidium* spp. were detected in the following five species: four round sardinellas, two wild European seabass, one Mediterranean horse mackerel, one blotched picarel, and one pompano (*Trachinotus ovatus*). Lastly, the only positive fish from the extractive fisheries group was a bogue (*Boops boops*). The mean total body length for each species in which positive individuals were detected indicated that the wild individuals analyzed were adults, the vast majority of them having reached sexual maturity. In the case of farmed species, although they were not sexually mature individuals in all cases, they could be considered young adults, with a minimum on-growing period in sea pens of approximately 20 months (**Table 4**).

Sequence and phylogenetic analyses at the 18S rRNA gene identified two species of *Cryptosporidium*, *Cryptosporidium molnari* (76.5%; 13/17) and *C. ubiquitum* (11.8%; 2/17), one isolate similar to *Cryptosporidium scophthalmi* (5.9%; 1/17), and one unidentified *Cryptosporidium* (5.9%; 1/17). Curiously, we also detected several unspecific amplifications (with slightly different size bands), showing the best match in GenBank with other protozoa (*Eimeria* spp., *Goussia* spp., *Calyptospora* spp.).

C. molnari was identified in the three groups studied and in seven different host species (**Table 4**). Nine out of 13 positive individuals identified as *C. molnari* (samples CS1–9) were 100% identical among them and exhibited a genetic identity (ID) of 99.80% with the sequence deposited in the GenBank under the accession number HM243550. This sequence diverged from isolates CS1–9 in a unique single nucleotide variant (SNV). Samples CS10–12 exhibited an ID between 99.50–99.61% with the same reference sequence and presented the same SNV together with others. Finally, one *C. molnari* isolated from an extractive fisheries specimen (sample FM1) was genetically closer to the sequence deposited in the GenBank under the accession number HQ585890 (**Table 5, Figure 12**).

Table 4. Presence of *Cryptosporidium* spp. and *C. molnari* for each species in which positive individuals were detected. Study group, mean weight, mean total body length, and reference total body length at sexual maturity are indicated.

Host species	N	Mean weight ± STD (g)	Mean TBL ± STD (cm)	Mean TBL at sexual maturity (cm)[a]	*Cryptosporidium* presence (%)	*C. molnari* presence (%)	Group
***Dicentrarchus labrax* (European seabass)**	84	384.51 ± 186.86	31.15 ± 5.52	36.1	4.76 (4/84)	3.57 (3/84)	C
	10	318.74 ± 85.83	33.36 ± 1.94		20 (2/10)	10 (1/10)	S
***Sparus aurata* (Gilthead seabream)**	39	279.25 ± 75.58	24.45 ± 2.13	36.5	5.12 (2/39)	5.12 (2/39)	C
	2	509.57 ± 312.47	31.6 ± 7.64		0	0	S
***Argyrosomus regius* (Meagre)**	24	474.68 ± 269.92	41.98 ± 11.99	61.6	4.17 (1/24)	0	C
***Sardinella aurita* (Round sardinella)**	25	96.84 ± 32.45	22.28 ± 2.47	18.8	16 (4/25)	12 (3/25)	S
***Boops boops* (Bogue)**	6	105.26 ± 40.79	21.5 ± 3.89	14.3	0	0	S
	3	76.56 ± 24.31	19.77 ± 2.42		33.34 (1/3)	33.34 (1/3)	EF
***Spicara maena* (Blotched picarel)**	11	77.05 ± 38.67	18.85 ± 3.77	11.5	9.09 (1/11)	9.09 (1/11)	S
***Trachinotus ovatus* (Pompano)**	2	139.64 ± 53.97	24.95 ± 1.48	30	50 (1/2)	50 (1/2)	S
***Trachurus mediterraneus* (Mediterranean horse mackerel)**	31	164.28 ± 63.57	26.47 ± 3.82	20	3.23 (1/31)	3.23 (1/31)	S
	3	65.65 ± 21.67	19.5 ± 2.18		0	0	EF

[a]Data extracted from: https://www.fishbase.se/search.php (accessed on 13 April 2022), González-Quiros et al. (2011), and Villegas-Hernández et al. (2016). C: cultivated fish; EF: fish from extractive fisheries; N: total number of specimens analyzed for each host species and group; S: synanthropic fish; STD: standard deviation; TBL: total body lenght.

Two sequences were identified as zoonotic *C. ubiquitum*, both in European seabass. One of them (sample CS14) presented 100% ID with the sequence deposited in GenBank under accession number MT044147. The other sample (CS13) exhibited 100% ID with a sequence from *Cryptosporidium* cervine genotype (GU124629), the former name for *C. ubiquitum* (**Table 5, Figure 12**). Attempts at amplifying these samples at the 60-kDa glycoprotein gene (*GP60*; Li et al., 2014) failed and isolates could not be subtyped.

One sequence from a cultivated meagre (sample CS15) showed 97.21% ID with *C. scophthalmi* reference sequence (KR340588). Phylogenetic analysis revealed a genetic distance of 4.8% (**Table 5, Figure 12**).

Finally, sample CS16 from one round sardinella was closer to *C. bollandi*, although it only presented 88.16% genetic similarity with this species reference sequence (MT169961). The genetic distance between sample CS16 and *C. bollandi* was 25.3% and phylogenetic analysis inferred a new clade for this sample, highly divergent from all the species and genotypes previously reported (**Table 5, Figure 12**).

From 17 positive isolates, five were successfully amplified and sequenced at the actin gene locus. For the following four samples, there was concordance with the 18S rRNA gene identification: samples CS4, CS8, and CS12 corresponded to *C. molnari*, while sample CS15 presented a nucleotide sequence more closely related to *C. scophthalmi* (genetic distance of 3.8%), as occurred at the 18S rRNA gene locus, although in this case the isolate clustered separately (**Figure 13**). Isolate CS9, which was typed as *C. molnari* at the 18S rRNA gene, was more closely related to *C. scophthalmi* at the actin gene (genetic distance of 2.7%), suggesting a probably mixed infection in this specimen (**Table 5**, **Figure 13**).

Partial sequences of the *Cryptosporidium* spp. 18S rRNA and actin genes obtained in this study were deposited in GenBank under accession numbers OM574856-OM574862 and OM650810-OM650814, respectively.

Table 5. *Cryptosporidium* spp. identified in marine fish at the 18S rRNA and actin genes.

Sample	Host Species	Group	Farm/ Market	18S rRNA			Actin		
				ID	Most Similar Sequence	% ID/SNVs	ID	Most Similar Sequence	% ID/SNVs
CS1	*Dicentrarchus labrax*	C	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS2	*Dicentrarchus labrax*	C	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS3	*Dicentrarchus labrax*	C	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS4	*Sparus aurata*	C	4	*C. molnari*	HM243550	99.80/1	*C. molnari*	HM365220	99.25/2
CS5	*Spicara maena*	S	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS6	*Sardinella aurita*	S	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS7	*Dicentrarchus labrax*	S	4	*C. molnari*	HM243550	99.80/1	---	---	---
CS8	*Trachurus mediterraneus*	S	1	*C. molnari*	HM243550	99.80/1	*C. molnari*	HM365220	98.83/3
CS9	*Sardinella aurita*	S	3	*C. molnari*	HM243550	99.80/1	*C. scophthalmi*-like	KR340589	95.77/33[a]
CS10	*Sparus aurata*	C	1	*C. molnari*	HM243550	99.50/3	---	---	---
CS11	*Sardinella aurita*	S	3	*C. molnari*	HM243550	99.60/2	---	---	---
CS12	*Trachinotus ovatus*	S	3	*C. molnari*	HM243550	99.61/2	*C. molnari*	HM365220	98.20/11
FM1	*Boops boops*	EF	2	*C. molnari*	HQ585890	99.80/1	---	---	---
CS13	*Dicentrarchus labrax*	S	2	*C. ubiquitum*	GU124629	100	---	---	---
CS14	*Dicentrarchus labrax*	C	2	*C. ubiquitum*	MT044147	100	---	---	---
CS15	*Argyrosomus regius*	C	2	*C. scophthalmi*-like	KR340588	97.21/14	*C. scophthalmi*-like	KR340589	96.11/21[b]
CS16	*Sardinella aurita*	S	4	Unidentified	MT169961	88.16/56	---	---	---

[a]Query cover: 95%. [b] Query cover: 92%. C: cultivated fish; EF: fish from extractive fisheries; ID: identity; S: synanthropic fish; SNV: single nucleotide variant. Symbol "—" means that actin gene couldn't be amplified for that sample.

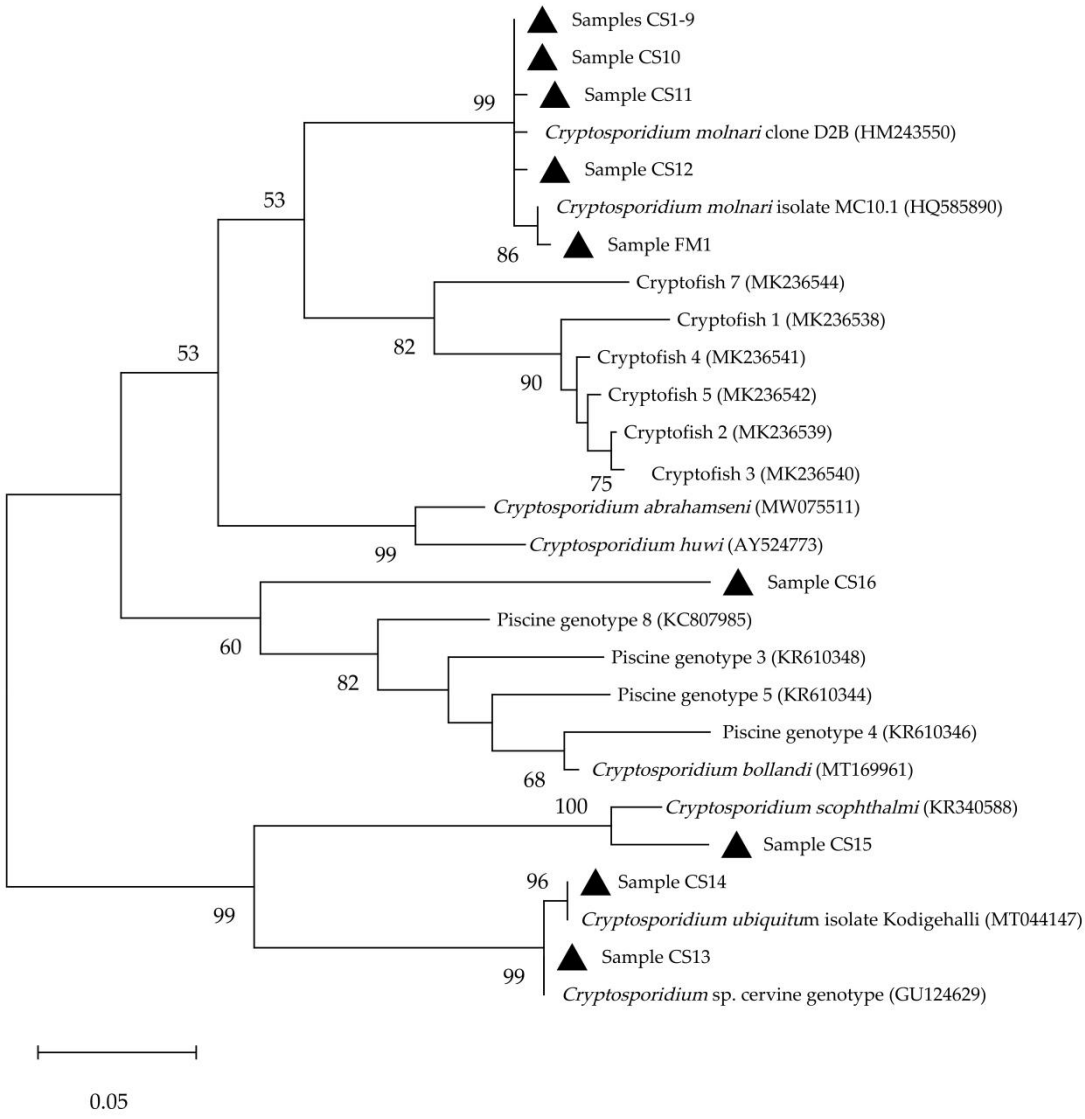

Figure 12. Phylogenetic relationships between *Cryptosporidium* isolates from this study (▲) and other *Cryptosporidium* species and genotypes inferred by Maximum-Likelihood (ML) method of 18S rRNA gene sequences (277 bp). Percentage support (>50%) from 1,000 replicates (bootstrap test) is indicated at the left of the supported node. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site.

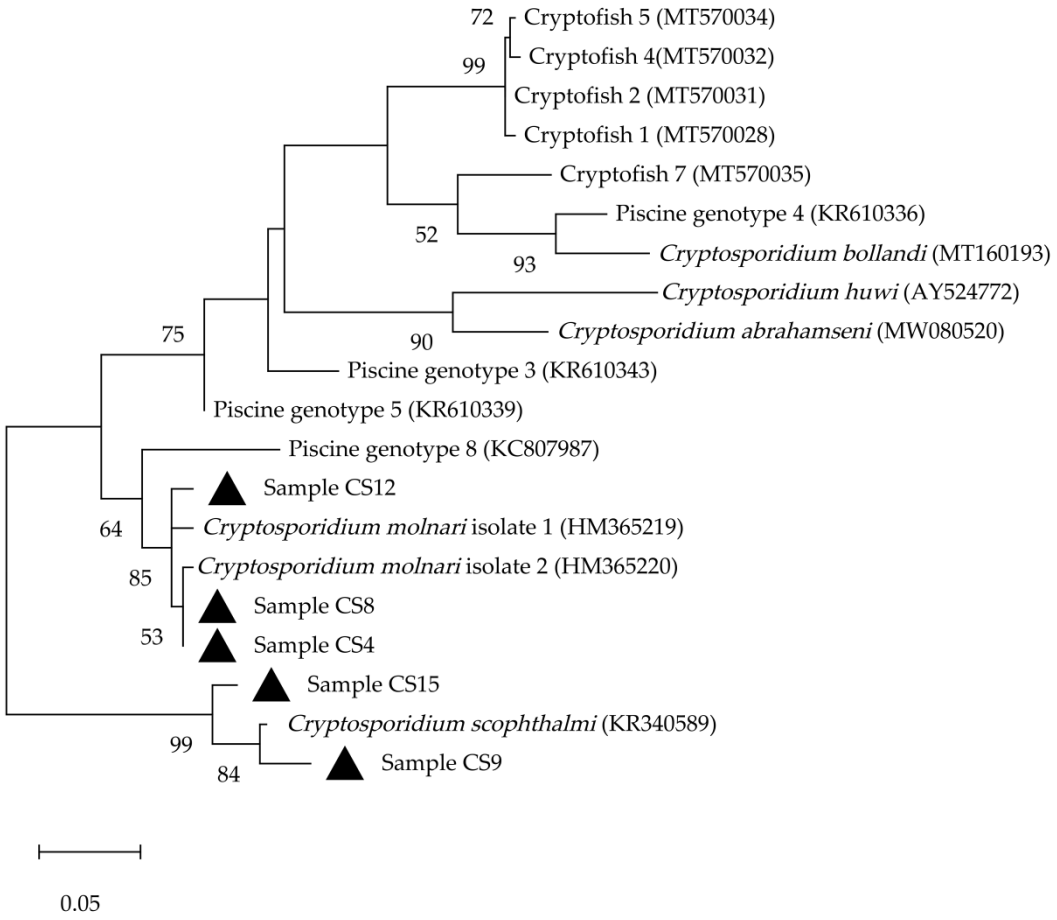

Figure 13. Phylogenetic relationships between *Cryptosporidium* isolates from this study (▲) and other *Cryptosporidium* species and genotypes from fish host inferred by Maximum-Likelihood (ML) method of actin gene sequences (192 bp). Percentage support (>50%) from 1,000 replicates (bootstrap test) is indicated at the left of the supported node. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site.

3.3. *Blastocystis* sp.

Blastocystis sp. was also analyzed on the three sampled fish groups: cultivated fish (N=147), synanthropic fish (N=147), and wild fish from extractive fisheries (N=110).

A general very low prevalence of 1.2 ± 0.5% (5/404) was detected, corresponding to five wild synanthropic fishes caught in the surroundings of the aquaculture facilities (3.4 ± 1.5%; 5/147). None of the cultivated fish nor the wild fish from extractive fisheries were positive. The positives from the synanthropic group corresponded to

two sparids, namely gilthead seabream and common two-banded seabream, one blotched picarel, and two round sardinellas.

Sanger-sequencing revealed three different STs: ST5, ST7, and ST15. Next generation amplicon sequencing was only successful in one sample, identifying a mixed infection of ST6 and ST7 (**Table 6**). Two samples failed at the library preparation step. *Blastocystis* sp. could not be detected for the other two samples.

The obtained *Blastocystis* sp. partial sequences for the 18S rRNA gene showed 100% query cover (QC) and 99.3-100% ID with already existing sequences. Therefore, phylogenetic analyses were not necessary.

As well as for *Cryptosporidium* spp., we detected unspecific reactions in both applied techniques. Amplified sequences showed the highest % ID with sequences deposited in NCBI GenBank as uncultured eukaryotes, diatoms, the protozoan *Eimeria* spp., and plants (*Brassica* spp.)

Table 6. Presence of *Blastocystis* sp. for each species in which positive individuals were detected. Subtypes identified are indicated.

Host species	**N**	***Blastocystis* sp. presence (%)**	**Subtypes**	
			Sanger	**NGS**
***Sparus aurata* (Gilthead seabream)**	2	50 (1/2)	ST5	---
***Diplodus vulgaris* (Common two-banded seabream)**	15	6.67 (1/15)	ST5	---
***Spicara maena* (Blotched picarel)**	11	9.09 (1/11)	ST7	---
***Sardinella aurita* (Round sardinella)**	25	8 (2/25)	ST7	ST7 + ST6
			ST15	---

NGS: next generation sequencing.

3.4. Microsporidia

In the case of microsporidia only two groups of fish were analyzed: cultivated fish (N=138) and wild fish from extractive fisheries (N=113).

Zoonotic microsporidia were detected in the gastrointestinal tract of 36 out of 251 analyzed fishes (14.3%). Of these, 30 fishes were wild fish from fish markets (26.6%; 30/113), and only six individuals were cultivated fish (4.4%; 6/138). The most prevalent was Enterocytozoonidae (9.9%; 25/251), followed by *E. intestinalis/hellem* (4.4%; 11/251); no co-infections were detected between both. All samples were negative for *E. cuniculi* (**Figure 14**). Positive isolates from farmed-raised fish were detected in European seabass and gilthead seabream from only two of the four sampled farms. Concerning wild fish, positives were detected in several species from the different fish markets (**Table 7**).

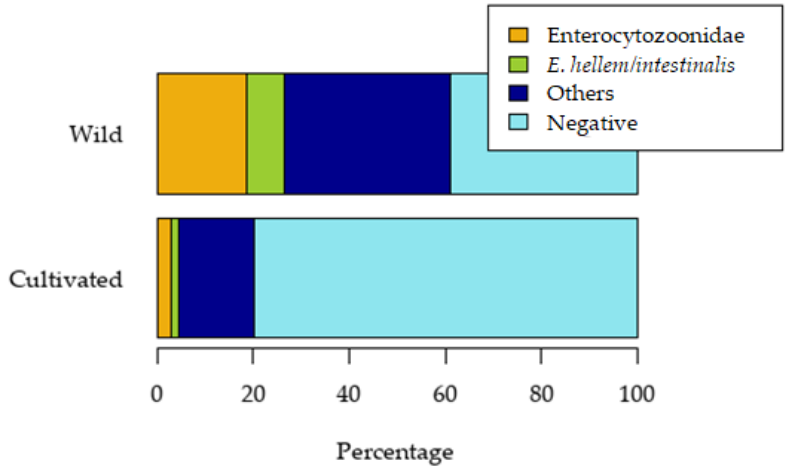

Figure 14. Presence of different microsporidia detected according to fish group.

From the 36 positive fish, the muscle was only available in 26 cases. None of the 26 analyzed samples were positive for Enterocytozoonidae or *E. intestinalis/hellem*.

Additionally, 61 out of 251 samples amplified at a Tm between 80.00 and 81.95 ºC. Amplification within this range potentially indicates the presence of other microsporidia species different from those targeted by the real-time PCR employed in this study. From among these samples, those with a cycle threshold (Ct) below 30 (N=18) were subjected to amplicon purification (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel) and subsequent Sanger-sequencing by Macrogen laboratories sequencing service (Korea). Twelve sequences of approximately 250 bp of the 18S rRNA were obtained, which showed good quality for further processing. Sequences

were blasted against microsporidia sequences from the NCBI GenBank database.

Table 7. Positives to zoonotic microsporidia species according to fish group and host species.

Group	Host species	N	*E. b*	*E. h/i*
Cultivated fish	*Argyrosomus regius* (Meagre)	23	0	0
	Dicentrarchus labrax (European seabass)	79	3	2
	Sparus aurata (Gilthead seabream)	36	1	0
		138	**4**	**2**
Wild fish	*Diplodus annularis* (Annular seabream)	2	1	1
	Helicolenus dactylopterus (Blackbelly rosefish)	1	1	0
	Lepidotrigla cavillone (Large-scaled gurnard)	1	1	0
	Merluccius merluccius (European hake)	12	3	0
	Micromessistius poutassou (Blue whiting)	5	2	0
	Mullus barbatus (Red mullet)	9	0	1
	Mullus surmuletus (Surmullet)	3	1	1
	Pagellus acarne (Axillary seabream)	1	0	1
	Pagellys eythrinus (Common pandora)	6	0	2
	Phycis blennoides (Greater forkbeard)	8	1	0
	Scomber scombrus (Atlantic mackerel)	22	8	0
	Serranus cabrilla (Comber)	1	1	0
	Serranus hepatus (Brown comber)	2	0	1
	Trachurus mediterraneus (Mediterranean horse mackerel)	4	1	1
		113	**20**	**8**

E.b.: *Enterocytozoon bieneusi; E. h/i*: *Encephalitozoon hellem/intestinalis*

Phylogenetic analysis was conducted using MEGA software, version 11 (Tamura et al., 2021). The obtained partial sequences were aligned with reference sequences from microsporidia species belonging to the same families/group as the reported sequences. The phylogenetic trees were inferred by ML method using the K2 + G substitution model. The analysis revealed two groups of homologous sequences and one different sequence. Group 1 (N=6) exhibited ≈93% ID with one isolate from the

decapod *Litopenaeus setiferus* (AJ252959). Although these authors found the closest similarity with *Pleistophora* sp., our phylogenetic reconstruction showed that it grouped with Pereziidae, a family of microsporidia infecting marine decapods, which clusters in the Glugeida group together with Pleistophoridae (Bojko et al., 2022) (**Figure 15**). Group 2 (N=5) exhibited 91-92% ID with a not characterized microsporidian isolated (EF672513) from the common bottlenose dolphin (*Tursiops truncatus*), clustering within the family Enterocytozoonidae (Park and Poulin, 2021) (**Figure 16**). Finally, the latest sequence (CL23) showed 93.31% homology with *E. nucleophila* (KF135645), a member of the Enterocytozoonidae family (**Figure 16**). Partial sequences of the 18S rRNA gene obtained have been deposited in GenBank under the following accession numbers: OR001667-OR001678.

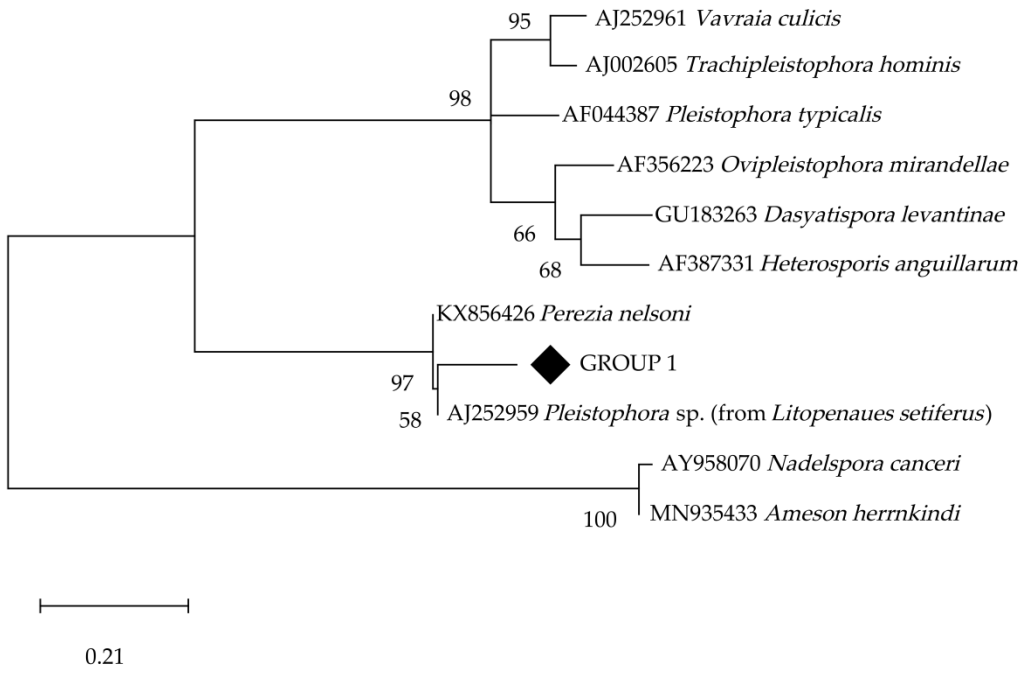

Figure 15. Phylogenetic tree of the other microsporidia reported in this study (◆) inferred by the Maximum-Likelihood (ML) method based on K2 + G substitution model applied to partial 18S rRNA gene sequences. Group 1 clustering in family Pereziidae, Glugeida group (273 bp). Percentage support from 2,000 replicates (bootstrap test) is indicated. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site.

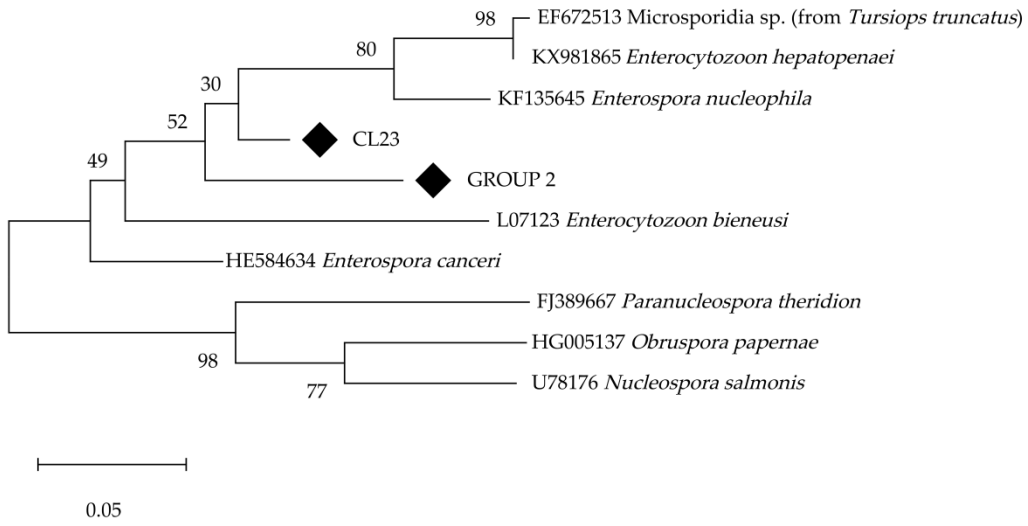

Figure 16. Phylogenetic tree of the other microsporidia reported in this study (◆) inferred by the Maximum-Likelihood (ML) method based on K2 + G substitution model applied to partial 18S rRNA gene sequences. Group 2 and sample CL23 clustering in family Enterocytozoonidae (198 bp). Percentage support from 2,000 replicates (bootstrap test) is indicated. The scale bar refers to a phylogenetic distance of 0.02 nucleotide substitutions per site

3.5. Coinfections

One coinfection was detected in a synanthropic blotched picarel between *C. molnari* and *Blastocystis* sp. ST5. Unfortunately, coinfections within microsporidia and the other two parasites could not be assessed, as positive samples for *Cryptosporidium* spp. and *Blastocystis* sp. could not be tested for microsporidia.

4. Discussion

4.1. *Cryptosporidium* spp.

The *Cryptosporidium* prevalence measured in adult wild fishes (3.9 ± 1.2%) can be considered low, with comparable results to those reported previously in Papua New Guinea (1.45%; Koinari et al., 2013), Australia (2.4%; Reid et al., 2010), and in different European seas (2.3–3.2%; Certad et al., 2019). The study of wild fish

allowed us to check not only commercial fishes from markets but also synanthropic fishes living in the vicinity of aquaculture farms. The inclusion of the synanthropic group could explain the slightly higher prevalence in the present study, due to higher population densities in the surroundings of aquaculture facilities, attracted by the abundance of food (Fernandez-Jover et al., 2007; Uglem et al., 2014). This fact could also explain the difference in prevalence detected between the synanthropic and extractive fishery groups (6.1% and 0.9%, respectively).

The *Cryptosporidium* spp. prevalence in the cultivated group was 4.8 ± 1.8%, corresponding to *C. molnari* infections in young adults of gilthead seabream and European seabass, and a *C. scophthalmi*-like isolate from a meagre. The prevalence obtained in gilthead seabream (5.12%) is comparable to the previously detected prevalence in individuals of similar weights in offshore on-growing systems (Sitjà-Bobadilla et al., 2005) and lower than the prevalence detected in younger fish, which are considered highly susceptible, as generally observed in human and animal cryptosporidiosis (Toledo et al., 2017; Levine et al., 2020). Prevalence in European seabass has been studied mainly among fingerlings and juveniles, with variable prevalence according to fish age and weight (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Sitjà-Bobadilla et al., 2005). Available data in older individuals reported 0% prevalence (Sitjà-Bobadilla et al., 2005; Certad et al., 2019). In the present study, only young adults from offshore on-growing farms have been sampled and analyzed, showing 3.57% prevalence.

The low prevalence in this survey could have been explained by the age of the fishes. A higher prevalence could be expected in younger individuals. However, fishes close to commercial size are more relevant for public health. In addition, future studies should consider the possibility of sampling throughout the year, to determine whether seasonality may influence prevalence.

Most positive fishes in this study corresponded to *C. molnari* isolates (76.5%), contrasting with previous surveys in marine fish, where different piscine genotypes

and zoonotic *C. parvum* were more frequent (Reid et al., 2010; Koinari et al., 2013; Certad et al., 2019). This could be explained by the fact that sampling comprised cultivated gilthead seabream and European seabass (type hosts for *C. molnari*), which were environmentally related to the synanthropic populations, facilitating the transmission of this parasite, as occurs in other parasitic infections (e.g., sea louse in Atlantic salmon; Costello, 2009; Nekouei et al., 2018). Therefore, *C. molnari* was detected in both cultivated species, but also in wild synanthropic round sardinella, blotched picarel, pompano, and Mediterranean horse mackerel, expanding the range of known hosts for *C. molnari*. Nine out of thirteen *C. molnari* isolates were homologous at 18S rRNA partial sequences obtained and differed in one SNV from other *C. molnari* available sequences. From those, seven samples corresponded to cultivated and synanthropic individuals from the same location (Farm 4, see **Table 5**); another two corresponded to wild synanthropic individuals captured in other locations (Farms 1 and 3), one Mediterranean horse mackerel and one round sardinella, both pelagic migratory species, commonly associated with marine farms (Fernandez-Jover et al., 2007). It is well known that coastal aquaculture facilities attract wild fish populations that forage on waste fish feed (Uglem et al., 2014). The movement of these farm-aggregating populations acts upon connecting farms and other marine areas (Arechavala-Lopez et al., 2010a; Arechavala-Lopez et al., 2010b; Uglem et al., 2014). This behavior could potentially enable the transmission of pathogens between farms and to wild populations through farm-aggregating wild fish movements (Uglem et al., 2014). Molecular data at the 18S rRNA gene from this study highlights the possibility of the spreading of *Cryptosporidium* spp. between different locations by migratory species that inhabit the surroundings of aquaculture facilities. Sequences at the actin gene for *C. molnari* isolates were in concordance with 18S rRNA data, except for a synanthropic round sardinella, which seemed to present a mixed infection with *C. molnari* (18S rRNA gene) and other isolates more similar to *C. scophthalmi* (actin gene).

No previous data exist for *Cryptosporidium* prevalence or species in meagre. In this study, only one individual out of 25 was found to be infected by one *Cryptosporidium* similar to *C. scophthalmi*. *C. scophthalmi* was originally described in cultured turbot (Alvarez-Pellitero et al., 2004) by microscopic examination and histological techniques. To date, the unique molecular data for this species has been reported by Costa and Saraiva (2015) in the same host. Isolate from meagre in this study exhibited a genetic distance of 4.8% (18S rRNA gene) and 3.8% (actin gene) with the turbot's sequences. It would be necessary to conduct a targeted study on meagres to better characterize this *C. scophthalmi*-like isolate.

C. ubiquitum was detected in two European seabass. One specimen belonged to the cultivated group while the other was part of the synanthropic group, both coming from the same location (Farm 2). It is important to highlight that the escape of fish from marine aquaculture farms has been reported around the world and in different cultivated species, including the European seabass (Toledo Guedes et al., 2009; Arechavala-Lopez et al., 2013). Therefore, we cannot exclude the possibility that synanthropic individuals of European seabass in this study were cultured individuals who had escaped from farms. To our knowledge, this is the first time that *C. ubiquitum* has been detected in a fish host. *C. ubiquitum*, formerly known as *Cryptosporidium* cervine genotype, is a widespread zoonotic emergent species, able to affect a wide range of hosts, greater than that of other *Cryptosporidium* species (domestic and wild ruminants, rodents, carnivores, and primates (Fayer et al., 2010). In Spain, *C. ubiquitum* reports are scarce, both in animals and humans. It has been reported in lambs and in an adult sheep (Diaz et al., 2015; Diaz et al., 2018), in a red fox (Mateo et al., 2017), and in a six-year-old child (Cieloszyk et al., 2012). Moreover, its presence in water sources has also been reported. In Spain, *C. ubiquitum* has been detected from an influent of a wastewater treatment plant (Galván et al., 2014). The presence of *C. ubiquitum* in water sources potentially explains its presence in fish hosts, as seems to occur with *C. parvum* (Certad et al., 2019). Zoonotic *Cryptosporidium* spp. can reach the marine environment from runoff or sewage

water, as they resist the disinfectants commonly used in the water industry (Galván et al., 2014). In this study, the sequences at the 18S rRNA gene of the two positive isolates were 100% identical to two sequences from domestic cattle from other countries. However, attempts to sub-type these isolates failed, making it difficult to identify a potential origin. The histopathological analysis would have been necessary to determine whether this was a possible natural infection or, on the contrary, the fish were only acting as mechanical transporters (Certad et al., 2015). However, the mere presence of *C. ubiquitum* in fish gastrointestinal tracts may pose a risk of transmission to humans. Although European seabass is not commonly consumed undercooked, it still remains a risk while handling (Graczyk et al., 2007). Moreover, other fish species, which are commonly consumed whole, raw or undercooked, could potentially harbor this zoonotic species.

Finally, sample C16 from a wild synanthropic round sardinella was identified as a *Cryptosporidium* sp. highly divergent from known species/genotypes. As inferred by the ML method at the 18S rRNA gene (**Figure 12**), it seems to constitute a new clade. Unfortunately, attempts to amplify the actin locus failed for this isolate. Although molecular data at the 18S rRNA gene could be indicative of a new *Cryptosporidium* species, it would be necessary to detect more isolates and perform more molecular and morphological studies.

4.2. *Blastocystis* sp.

We got a very low prevalence (1.2 ± 0.5%) in the present survey, lower than the obtained in the only previous study conducted on marine fish (3.5%; Gantois et al., 2020). The study of Gantois et al. (2020) was conducted in different waters, the Northeast Atlantic, and encompassed only four species obtained from fishing activity. These authors noticed differences in prevalence according to the species, with herring (*Cuplea harengus*) having a four-fold higher risk of testing positive than Atlantic mackerel, whiting (*Merlangus merlangus*), and saithe (*Pollachius virens*). In contrast, we surveyed three different marine fish groups (cultivated fish, wild fish

from fisheries, and synanthropic wild fish) from the western Mediterranean, and all the positives were detected in the last group (3.4 ± 1.5%). Among the targeted species, only the Atlantic mackerel was coincident with the species analyzed in Gantois et al. (2020), although our sample size was smaller (N=22) and no positives were detected. However, two out of the five positive samples corresponded to round sardinellas (2/25; 8%), which is similar to the prevalence obtained for herring (5/60; 8.3%) by Gantois et al. (2020), both species belonging to the same family Cupleidae.

To our knowledge, only two other studies have surveyed *Blastocystis* sp. in fish, but freshwater species. König and Müller (1997) found a prevalence of 11% (2/18) while Rauff-Adedotun et al. (2022) did not detect any positives (0/123); both studies relied on the cultivation of the samples and did not provide information on STs.

Four out of five positive isolates corresponded to STs with known zoonotic potential (ST5, ST6, and ST7), with the remaining one matching ST15. Gantois et al., (2020) also detected zoonotic STs, mostly ST2 and ST8, and only one positive for ST7 (a herring). Additionally, they detected several untypable poikilotherm isolates, corresponding to potential new STs specific to fish. Curiously, we did not detect any poikilotherm-derived isolate during this survey.

ST5 was detected in the two positive sparids. Pigs seem to be the primary reservoir for this zoonotic ST, also in Spain (Asghari et al., 2021; Rivero-Juarez et al., 2020), with sporadic infections in humans (Wang et al., 2014). The low prevalence detected in fish suggests that they are not natural hosts, but acquire this *Blastocystis* ST by seawater contamination with terrestrial animal or human sewage. ST7 was detected in two specimens, a blotched picarel and a round sardinella, the second presenting a mixed infection with ST6. Both ST6 and ST7 are predominant in avian hosts (Cian et al., 2017; Barati et al., 2022), although transmissible to humans (Greige et al., 2018); therefore, their scarce presence in fish is more likely to be due to seawater contamination by bird or human feces. Finally, ST15 detected from one round

sardinella is an ST commonly found in non-human primates and, to a lesser extent, in Artiodactyla (Cian et al., 2017); again we suggest that fish are not natural hosts for this ST and probably acquired it from seawater contamination. Information on *Blastocystis* sp. presence in water is still scarce. To date, there are no reports of any of these STs in seawater, although ST5, ST6, and ST7 have been punctually reported from other water sources in Iran, Malaysia, and Thailand (Attah et al., 2022). In Spain, there is one report of ST2 and ST4 in irrigation waters (Moreno et al., 2018) and ST1-ST4, ST6, and ST8 in reused wastewater (Moreno-Mesonero et al., 2022).

The primers used for *Blastocystis* sp. detection (Poirier et al., 2011) amplify a region comprised within positions 18 and 339 of the 18S rRNA gene (using ST7 as reference). On the other hand, primers used for next generation amplicon sequencing (Maloney et al., 2019) amplify a region of the same gene, between 445-464 and 905-924 positions. Both primers amplify a region that provides enough information for subtyping and show high sensibility and specificity (Santín et al., 2011; Cian et al., 2017). However, in the present study, when next generation sequencing amplicon was applied, we failed to amplify (2/5) or detect *Blastocystis* sp. (2/5) in most of the samples. This difference when applying different protocols to the same sample could be due to differences in sensibility between the two primer sets.

4.3. Microsporidia

We detect the presence of zoonotic microsporidia species *E. intestinalis/hellem* in marine fish for the first time. It is well-known that water plays an essential role in microsporidian spores' survival and transmission to humans, either for direct consumption, food irrigation, or recreational bathing (Thurston-Enriquez et al., 2002; Fayer and Santin-Duran, 2014). Therefore, fish could get spores from marine water contaminated by infected humans and animals.

We utilized a SYBR green real-time PCR, previously described by Polley et al. (2011), which allows the simultaneous detection and species identification of the

main human pathogenic microsporidia species. These assays are appropriate when the expected parasite load is low, as is foreseeable in the case of fish acting as passive carriers. They offer better sensitivity compared to conventional PCR techniques and simultaneously enable multiple species testing, facilitating a comprehensive analysis even when limited DNA samples. However, the limitation of this method is that it does not allow for genotyping of the isolates.

The highest presence (9.96%) was detected for Enterocytozoonidae, potentially *E. bieneusi*, which is the most common microsporidian species diagnosed in humans (Matos et al., 2012), but also present in a wide range of birds and mammals (Haro et al., 2005; Santín and Fayer, 2011). Interestingly, this species groups in a family of microsporidians, whose other components infect only aquatic hosts (fish and crustaceans) (Stentiford et al., 2013; Park and Poulin, 2021). *E. bieneusi* has also been detected in coastal waters (Graczyk et al., 2010; Moss and Snyder, 2019) and in filter-feeding mollusks (e.g., Lucy et al., 2008). Nevertheless, the question remains about their role: authentic hosts or passive carriers. Similarly, we are reporting the first evidence of the potential presence of *E. bieneusi* in fish hosts, although we also cannot discern whether an infection is occurring or, if on the contrary, fish got the parasite in their digestive tracts from the water and/or by the feeding on aquatic invertebrates. Regardless, the close phylogenetic relationship between *E. bieneusi* and other aquatic microsporidians enhances the interest in elucidating the epidemiological role that water and aquatic hosts could play in *E. bieneusi* human infections. In this regard, it is essential to consider the enormous genetic variability exhibited by *E. bieneusi*. Based on the analysis of the ribosomal internal transcribed spacer (*ITS*) region, several genotypes of *E. bieneusi*, distributed in eleven groups and with different host specificities, have been described. Zoonotic genotypes from group 1 are among the most frequently reported in different water sources (Li et al., 2019). Further studies delving deeper into *E. bieneusi* genotyping in aquatic hosts should be addressed to ascertain the role that seafood and, in particular, fish, might play in *E. bieneusi* epidemiology and public health.

A presence of 4.38% was detected for zoonotic *E.intestinalis/hellem*. *E. intestinalis* is the second most common cause of microsporidian diarrhea in humans, only behind *E. bieneusi* (Weiss, 2014), and, interestingly, it has been identified in recreational water rivers from northern Spain (Izquierdo et al., 2011). *E. hellem* causes disseminated infections and has not been associated with diarrheal syndrome. It is commonly associated with keratoconjunctivitis, and also affects respiratory and urinary systems (Izquierdo et al., 2022). Conversely to Enterocytozoonidae, both these species pertain to a family whose members are primarily terrestrial (Bojko et al., 2022), suggesting that, in this case, fish are most probably acting as passive carriers.

The other *Encephalitozoon* species tracked in this survey, namely *E. cuniculi*, has not been detected in any of the sampled fishes, although it is also present in water environments (Galván et al., 2013; Moss and Snyder, 2019). *E. cuniculi* is the fourth most common microsporidian species found in humans. Although it can occasionally cause intestinal disease, it is most commonly found affecting the brain and kidneys (Izquierdo et al., 2022).

Although we retrieved other microsporidia sequences from this study, their phylogenetic classification must be interpreted cautiously, as it relies on short fragments of the 18S rRNA gene. Nonetheless, the information provided is in congruence with the samples' nature, encompassing these sequences into two families of marine microsporidia, i.e., Pereziidae and Enterocytozoonidae.

Notably, a high presence was detected in wild fishes (26.55%) compared to a low presence in the cultivated group (4.35%). Open-net pens limit farmed fish space, which prevents them from foraging in their natural habitat. Hence, their nutrition relies almost exclusively on provided extruded feed, except for the eventual ingestion of small crustaceans and mollusks that can go through the net (Skov et al., 2009). On the other hand, wild fishes are unrestricted in their movements and get food from the natural environment. Therefore, wild fish commonly feed on

shellfish, which can serve as reservoirs and carriers for these zoonotic microsporidia (Graczyk et al., 2004; Lucy et al., 2008). Additionally, wild fish are more likely to come into contact with polluted areas where various contaminants, including pathogens like microsporidia, can be present (e.g., sewage discharges; Cheng et al., 2011).

The absence of Enterocytozoonidae and *E. intestinalis/hellem* in the muscular tissue of positive fish is under the hypothesis that fish act merely as passive carriers. Even if they were natural hosts, these species commonly affect the gastrointestinal tract and other inner organs (Izquierdo et al., 2022). Despite this, there is still a potential risk for the consumer due to cross-contamination during handling and evisceration (Graczyk et al., 2007; Chintagari et al., 2017), moreover if we consider that the consumption of raw and undercooked fish dishes has notably increased in recent years in western countries (Broglia et al., 2011).

CHAPTER 2

CHAPTER 2. Unraveling parasites by exploratory NGS on *Sardina pilchardus* and *Sardinella aurita* (Cupleidae) gastrointestinal samples: a pilot study

1. Introduction

Parasitism is considered one of the most successful modes of life on Earth, representing as far as 40% of the overall organisms (Dobson et al., 2008). However, estimations point out current poor knowledge of the real global parasite richness and distribution (Larsen et al., 2017; Okamura et al., 2018). The study of parasite diversity gained importance in recent years, both for their ecological significance and importance in conservation and because of the emergence of parasitic diseases in the livestock-wildlife-human interface (Carlson et al., 2020).

Traditionally, studies on parasite communities have relied on host dissection, parasite isolation, and morphological identification. This approach requires extensive taxonomic expertise and it is time-consuming. Moreover, morphological characteristics may not be sufficient for classification, especially at the species level. PCR amplification plus sequencing can be useful for higher taxonomic resolution, but requires previous knowledge of the expected parasite community; it is also time-consuming and cost-expensive when several parasites and samples need to be assessed (Tanaka et al., 2014; Hino et al., 2016). Because of these constraints, surveys employing both methods usually focus on a single parasitic taxon (class, family, or even in a specific genus) (Scheifler et al., 2019). As a result, the knowledge of parasite communities is often incomplete and ambiguous (Hino et al., 2016).

The advent of high-throughput sequencing or next generation sequencing (NGS) methods, together with the use of wide coverage or universal primers and the development of bioinformatics, has revolutionized the research on microbial communities. Indeed, the metabarcoding -a technique that employs high-throughput DNA sequencing to simultaneously identify multiple organisms within a complex matrix based on specific DNA markers- of the 16S ribosomal RNA (16S rRNA) gene has arisen as a common and reliable technique for assessing microbial diversity in an extensive range of biomes (Nayfach et al., 2021). However, high-throughput metabarcoding application to eukaryotic diversity is still lagging

behind. It has been mostly applied for dietary studies and to assess the biotic composition of ecosystems based on environmental DNA, especially relevant in aquatic environments (Cristescu and Hebert, 2018; de Sousa et al., 2019). The application of this method for characterizing parasite communities is currently emerging, and metabarcoding surveys of parasites have been mainly applied to specific taxons, such as protozoans or helminths (Tanaka et al., 2014; DeMone et al., 2020). The conducted studies present this new sequencing approach as an alternative technique to overcome the abovementioned issues inherent to traditional methods. It is a faster and relatively low-coast technique, compared to morphological and targeted PCR identification, that requires less taxonomic expertise and allows the simultaneous classification of different parasites present in several samples (Tanaka et al., 2014; Hino et al., 2016).

Marine fishes are hosts of a plethora of parasitic organisms (Klimpel et al., 2019) that impact host fitness and behavior, which in turn has important implications on population dynamics and ecosystems (Hatcher et al., 2012). Along with the ecological importance, there is also the public health perspective, since some of these parasites are known to be zoonotic, posing a health risk through fish consumption. Additionally, fish can also carry other human pathogenic parasites from terrestrial origin (see Introduction, sections 2 and 3, and Chapter 1). To date, metabarcoding surveys of parasites in fish hosts have scarcely been applied. Scheifler et al. (2019) analyzed the unspecific reads from a previous bacterial survey employing 16S rRNA primers in Mediterranean fish. These authors demonstrated the possibility of exploiting already existing data from previous bacterial metabarcoding projects for the purpose of assessing parasite diversity. Recently, Scheifler et al. (2022) applied a targeted metabarcoding approach to survey the ectoparasitic monogenean in sparid gills and skin.

We propose that general eukaryotic metabarcoding approaches used for dietary studies, one of the most common applications in marine organisms, could be useful for studying the gastrointestinal parasitic community of marine fishes, including the

detection of potentially zoonotic parasites and overcoming the issues that common morphological and PCR techniques present. Therefore, in the present study, we analyzed the gastrointestinal "parasitome" of two teleost fish species, the Mediterranean European pilchard (*Sardina pilchardus*) and the round sardinella (*Sardinella aurita*) (Cupleidae), using a metabarcoding approach intended for the dietary analysis of marine fish (Homma et al., 2022), which targets the V8-V9 variable region of the eukaryotic 18S rRNA gene. European pilchard and round sardinella are small pelagic species of great relevance in the ecological processes of pelagic marine ecosystems (Cury et al., 2000), as well as being of huge economic importance in the Mediterranean basin, mainly the European pilchard. Indeed, the European pilchard has been the most fished species in the western Mediterranean subregion, accounting for 18.2% of the total landings (period 2018-2020; FAO, 2022b). Interestingly, this species can harbor zoonotic parasites and is commonly consumed raw or undercooked in western Mediterranean countries, including Spain, posing a public health risk for consumers (Bušelić et al., 2018; Certad et al., 2019). The sympatric round sardinella has lower commercial value, with *Sardinella* spp. taking the third position in western Mediterranean total landings (FAO, 2022b). However, this species is expanding its distribution northwards (due to the increasing sea surface temperature) (Sabatés et al., 2006), and therefore becoming increasingly common.

This study aims to describe the gastrointestinal parasitic community of both species by employing a general eukaryotic metabarcoding approach and to detect the presence of zoonotic parasites. Additionally, we compared the results between this technique and conventional PCR methods for the parasites *Cryptosporidium* spp. and *Blastocystis* sp. (as employed in Chapter 1).

2. Material and methods

2.1. Fish sampling

Mediterranean European pilchards, hereafter pilchards, were purchased from

different supermarkets and fishmongers. All of them came from Spanish ports, whose fleets fish in waters belonging to the FAO zone 37.1.1 (from Girona (Cataluña) to Málaga (Andalucía), Spain). With some exceptions, all of them had been fished with purse-seine. The sampling was conducted during the so-called pilchard season (May to September 2022). An overall of 216 specimens were acquired from the different sale establishments. At the time of purchase, the exact origin was registered.

Sampled pilchards were stored in refrigeration and processed within the first 24-48 h after death; the time-lapse was variable due to the time passing between fishing activity and the sale. Body weight and total body length were registered and dissection was performed using sterile dissection material. A sample was taken consisting of gastrointestinal tissue scraping mixed with intestinal content for each individual and kept frozen at -20 ºC until further processing.

As for the round sardinellas, hereafter sardinellas, we used the samples obtained for the Chapter 1 study (N=25). Therefore, sardinellas came from synanthropic populations inhabiting the surroundings of offshore fattening farms. These samples were already processed, so the starting material was the genomic DNA (preserved at -20 ºC).

2.2. DNA extraction and targeted detection of *Cryptosporidium* spp. and *Blastocystis* sp. in the European pilchard

DNA extraction from pilchards' gastrointestinal samples, as well as molecular detection for *Cryptosporidium* spp. and *Blastocystis* sp. were performed according to the methodology described in Chapter 1.

2.3. Metabarcoding assay

The metabarcoding assay was performed in the sardinellas (N=25) and in a subsample of the pilchards (N=22).

For the purpose of studying the gastrointestinal parasites of these species, we applied a metabarcoding protocol using high throughput sequencing developed by Homma et al. (2022). This protocol targets the V8-V9 variable region of the eukaryotic 18S rRNA gene and includes a blocking primer specifically designed to suppress the amplification of fish 18S rDNA. The analysis, including protocol standardization, was performed by Sequencing Multiplex S.L. (Paterna, Valencia, Spain). The standardization included testing the effectiveness of the blocking primer in our targeted species. For this purpose, five individuals of each fish species were analyzed per duplicate, with and without blocking primer, and the percentage of host reads (Teleostei) was assessed.

PCR was performed using the eukaryote universal primer Vf8 (5'-ATAACAGGTCTGTGATGCCCT-3'; Bradley et al., 2016) and the universal reverse primer 18SVR9 designed by Homma et al., (2022) (5'-CCTTGTTACGACTTYTMCTTCCTCTA-3'), in combination with the blocking primer BlockFish_long6 (also designed by Homma et al. (2022); 5'-CCTCTAGATAGTCAAGTTTGATCGTCTTCTCGGC-3'). The library was prepared using the Illumina Metagenomic Sequencing Library Preparation protocol (Part# 15044223 Rev. B), including a dual indexing strategy. The PCR was performed on 25 µl total volume, including 10 ng of targeted DNA, 0.2 µM of each primer, and 2 µM of blocking primer. Cycling conditions were: 94 ºC for 15 min, 30 cycles of 94 ºC for 150 s, 66 ºC for 30 s, and 60 ºC for 1 min, and a final elongation consisting of 60 ºC for 10 min. Afterward, samples were analyzed using the QIAxcel Advanced System (Qiagen, CA, USA) and libraries were quantified using Qubit Fluorometric Quantitation assay (Thermo Fisher Scientific, MA, USA). A final pooled library concentration of 5 pM with 20% PhiX control was sequenced on an Illumina MiSeq using 500 cycle v2 chemistry (250 base pair, paired-end reads) (Illumina, CA, USA).

2.4. Bioinformatics and data analysis

Bioinformatic analysis was performed in collaboration with Marbyt – Smart

Solutions for Biotechnology, S.L. (Murcia, Spain). After demultiplexation and primers removal using Cutadapt (Martin, 2011), sequences were subjected to a quality analysis using FastQC version 0.11.9. Software QIIME 2 (Andrews, 2010; Bolyen et al., 2019) was used to further process the pair-end sequences. Denoising was performed using DADA2 algorithm (Callahan et al., 2016). At this step, and according to the FastQC analysis, truncation was made, being more restrictive with reverse sequences which showed worse quality (--p-trunc-len-f 187 y –p-trunc-len-r 130). Clustering was performed with VSEARCH algorithm (Rognes et al., 2016) following the open reference strategy. Finally, classification and taxonomy assignation of the obtained OTUs (Operational Taxonomic Units) was made using SILVA 138.1 release for eukaryotic 18S rRNA as reference database (Quast et al., 2013; Yilmaz et al., 2014; downloaded from https://data.qiime2.org/2023.7/common/silva-138-99-nb-classifier.qza) and the Naive Bayes classifier algorithm of SKLearn. For those OTUs pertaining to parasitic taxa that did not reach genus or species level assignation, we used the BLAST tool (Altschul et al., 1990) following the strategy employed by Scheifler et al. (2019). When deeper taxonomic classification was not possible, the obtained OTU classification was retained. Further data processing, plotting, and statistical tests were performed on R software version 4.1.2 (R Core Team, 2021), with the use of the following packages: ggplot2 3.4.3 (Wickham, 2016), reshape 0.8.9 (Wickham, 2007), dplyr 1.1.2 (Wickham et al., 2023), forcats 1.0.0 (Wickham, 2023), and rstatix 0.7.2 (Kassambara, 2023).

3. Results

3.1. Targeted detection results

Very low prevalences for *Cryptosporidium* spp. (0.93%; 2/216) and *Blastocystis* sp. (0.93%; 2/216) were detected in the pilchards. The obtained partial sequences for *Cryptosporidium* spp. were incomplete and showed very low quality; one of them could be potentially assigned to *Cryptosporidium andersoni.* Concerning *Blastocystis*

sp., Sanger sequencing revealed two subtypes, namely ST4 and ST5.

As regards the sardinellas, results on *Cryptosporidium* spp. and *Blastocystis* sp. presence were available from the study in Chapter 1. Briefly, four sardinellas were positive for *Cryptosporidium* spp. (*C. molnari*, N=3; unidentified like-*Cryptosporidium*, N=1), and two were positive for *Blastocystis* sp. (ST6+ST7 and ST15, respectively).

3.2. Blocking primer effectivity

We demonstrated the effectiveness of the blocking primer in both species. The proportion of host reads was reduced by 95.94% and 89.92% on average in the pilchards and sardinellas, respectively, when the blocking primer was used (**Figure 17**). It was noticeable that the proportion of host reads was very variable between individuals of the same species.

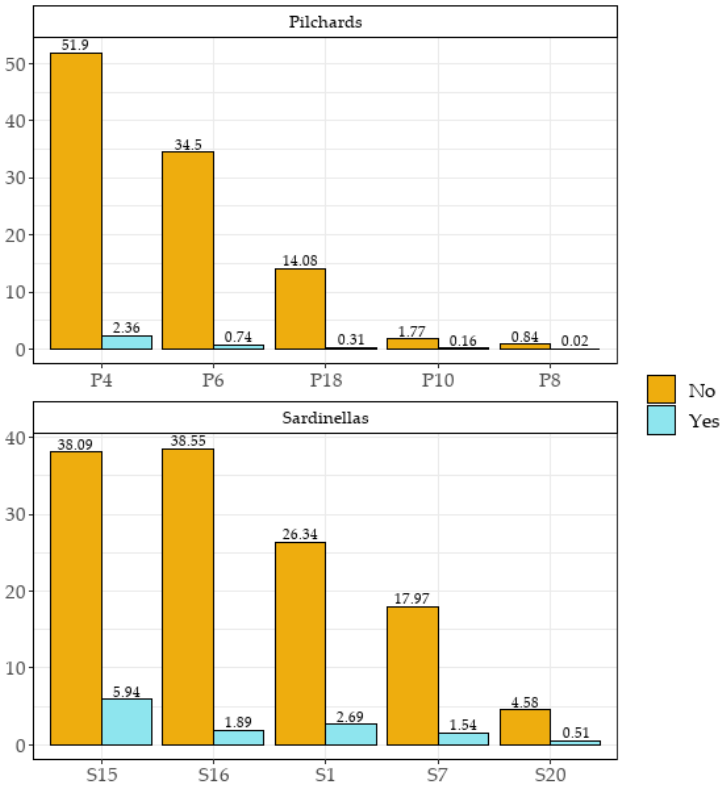

Figure 17. Percentage of host reads (i.e., Teleostei) with (Yes) and without (No) blocking primer.

3.3. Parasite community characterization by metabarcoding

Four out of 25 sardinellas' samples failed at the library generation step and were discarded from the study. The 22 pilchards were successful at this step. Therefore, the metabarcoding assay was conducted on 43 specimens, 21 sardinellas and 22 pilchards.

An overall of 7,932,005 reads were obtained, with 184,465 ± 78,935.5 (mean ± standard deviation) reads on average per sample. After the denoising step, 5,467,287 reads were retained, with 127,146.21 ± 63,307.66 reads on average per sample (i.e., 70.39 ± 18.2%). The mean length of the retained sequences was 296.18 ± 14.47 bp. Two samples, one pilchard and one sardinella, were discarded at this point because only 8% and 6.6% of reads, respectively, were retained; hence, 41 samples continued in the analysis. Finally, we discarded the OTUs that met one or more of these conditions: OTUs with "Unassigned" taxonomy, OTUs classified as "Eukaryota" without deeper taxonomic assignation, OTUs corresponding to remaining host DNA ("Teleostei"), OTUs with <5 reads, and OTUs that were present in a single sample with <30 reads. 929 OTUs were retained for further analysis (from an initial number of 2,218).

For each species, we calculated the relative abundance of each eukaryotic supergroup (Adl et al., 2019) from the overall of the retained eukaryotic reads. Members of Archaeplastida, Opisthokonta, and SAR (Stramenopiles, Alveolata, and Rhizaria), were detected in both species. The Holozoa, within the opisthokonts, was the most abundant group in both species (70.44 and 46.06% in pilchards and sardinellas, respectively) (**Figure 18**).

To characterize the gastrointestinal parasitic community, we focused on the OTUs that corresponded with taxa encompassing marine fish parasitic genera. Additionally, parasites potentially coming from dietary components (mostly prey) were also identified (**Supplementary table 4**). The remaining OTUs from non-parasitic organisms were removed from the analysis.

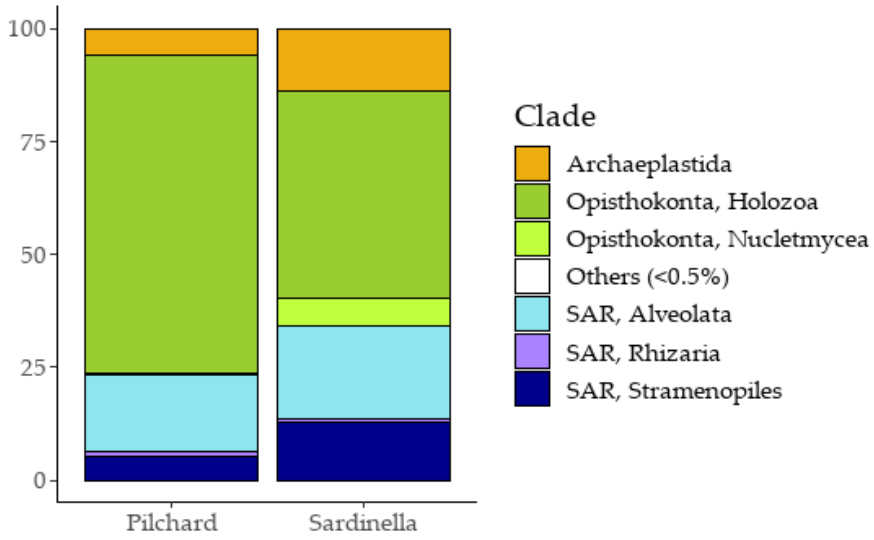

Figure 18. Relative abundance (proportion of reads) of each eukaryotic supergroup according to fish species. The category "Others" encompasses: Haptista and Telonemia, in sardinellas; and Cryptista, Haptista, and Picozoa, in pilchards.

Fish parasites were detected in 100% of pilchards and 85% of sardinellas. Parasites corresponding to the classes Trematoda, Conoidasida, and Myxozoa, were detected in both species, while Cestoda was only detected in pilchards. Trematoda (90.48% and 70%) and Conoidasida (90.48% and 65%) were the most prevalent in pilchards and sardinellas, respectively. Significant differences in parasitic presence between sardinellas and pilchards were detected only for Myxozoa and Cestoda (Fisher's exact test with Benjamini-Hochberg FDR correction, $p < 0.05$) (**Figure 19**). Pilchards showed significantly greater parasitic abundance for the overall parasite abundance and the Myxozoa and Cestoda relative abundances (Wilcoxon test with Benjamini-Hochberg FDR correction, $p < 0.05$). It is remarkable the high dispersion observed for Trematoda in sardinellas (**Figure 19**).

We identified eight genera (six species) within three out of the four abovementioned parasitic classes. It was not possible to go deeper into the Cestoda taxonomy classification. Species assignation was made when BLAST confirmed >99% ID (**Table 8**, **Figure 20**). From those, two genera/species are considered to be potentially zoonotic, namely *Cryptosporidium* sp. and *Kudoa thyrsites* (**Table 8**).

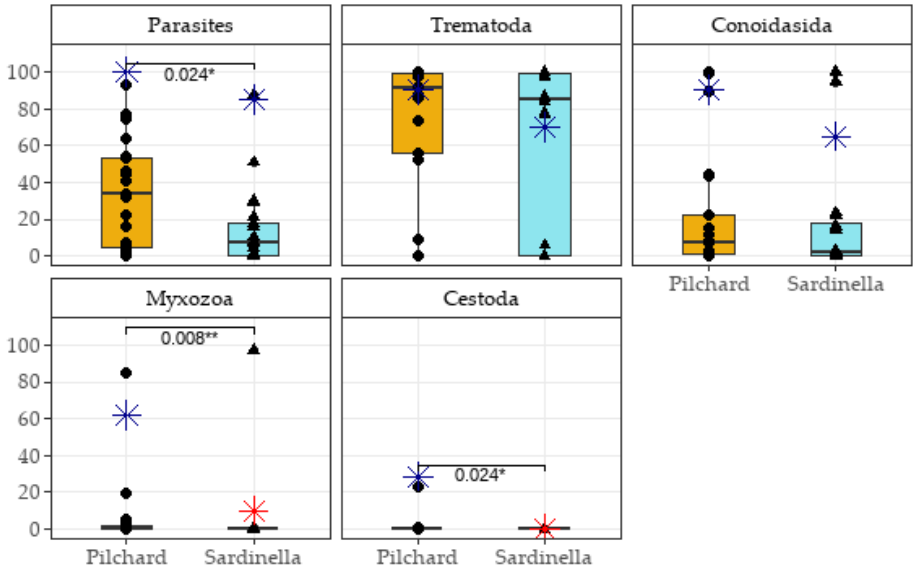

Figure 19. Boxplots (plus dot plots) comparing parasite reads abundance distribution between both species. Parasites: proportion of reads corresponding to fish parasites from the overall eukaryotic reads. Trematoda, Conoidasida, Myxozoa, and Cestoda: relative proportion of reads corresponding to each parasitic class from the overall parasitic reads. Significant differences are shown. The stars represent the proportion of individuals parasitized for each fish species, with red color indicating significant differences.

Table 8. Fish parasites identified at the level of genus or species.

Phylum, Class	Genus or Species	Number of reads	European pilchards (+/total)	Round sardinellas (+/total)	Zoonotic potential
Apicomplexa, Conoidasida	*Cryptosporidium*	109	4/21	1/20	Yes
	Goussia	77,258	9/21	11/20	No
	Goussia amealiae	37,715	17/21	0/20	No
Cnidaria, Myxozoa	*Ceratomyxa*	1,115	6/21	0/20	No
	Ceratomyxa diplodae	825	0/21	1/20	No
	Ceratomyxa sargus n.sp.	79	0/21	1/20	No
	Kudoa thyrsites	383	2/21	0/20	Yes
	Sphaerospora dicentrarchi	42	0/21	1/20	No
Platyhelminthes, Trematoda	*Aphanurus*	424,360	14/21	9/20	No
	Didymozoon	506	0/21	4/20	No
	Maccallumtrema xiphiados	1,759	0/21	2/20	No

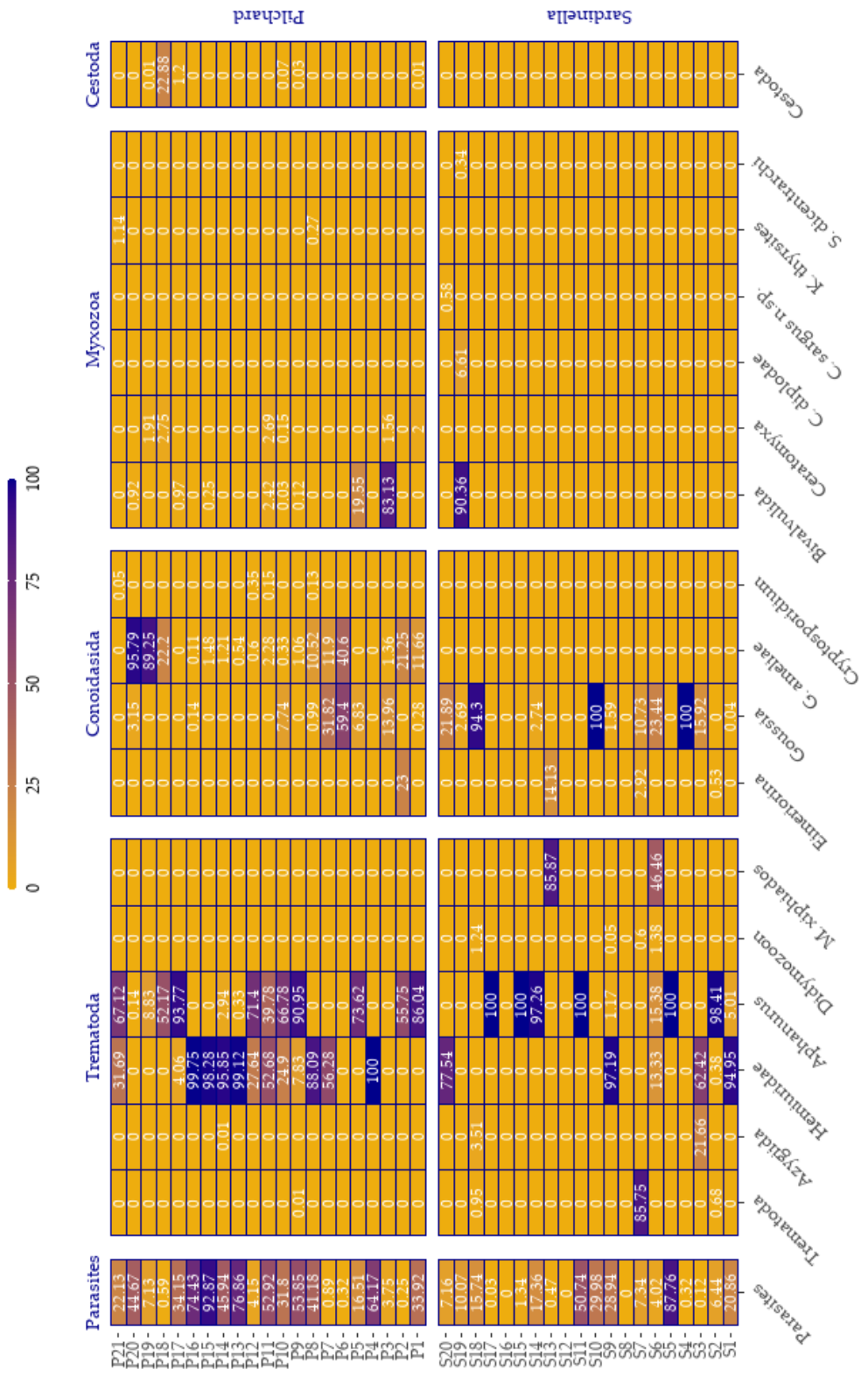

Figure 20. Heatmap showing the proportion of parasitic reads for each individual. The first column (Parasites) shows the proportion of reads corresponding to fish parasites from the overall eukaryotic reads. The following columns show the relative proportion of reads corresponding to each parasitic taxon from the overall parasitic reads.

Cryptosporidium was detected by this metabarcoding assay, but the results were not fully consistent with those obtained by PCR plus Sanger sequencing (**Table 9**). *Blastocystis* sp. and *Blastocystis phytoni* were each detected in one sardinella, but in four and nine reads respectively, and were therefore excluded from the main analysis. In addition, the samples did not match the PCR positive fish (**Table 9**).

Table 9. Comparison of results for *Cryptosporidium* and *Blastocystis* detection between PCR plus Sanger sequencing and the metabarcoding assay. Only positive specimens are shown.

Parasite	Fish species	Fish code	Sanger sequencing result	Metabarcoding result
Cryptosporidium	**Sardinella**	S9	*C. molnari*	NI
		S18	*C. molnari*	NI
		S20	*C. molnari*	NI
		S5	Unidentified like-*Cryptosporidium*	NI
	Pilchard	P8	*C. andersoni*	*Cryptosporidium* sp.
		P12	*Cryptosporidium* sp.	*Cryptosporidium* sp.
		P11	NI	*Cryptosporidium* sp.
		P21	NI	*Cryptosporidium* sp.
Blastocystis	**Sardinella**	S12	ST6+ST7	NI
		S17	ST15	NI
		S4	NI	*Blastocystis* sp.[*1]
		S13	NI	*Blastocystis phytoni*[*1]
	Pilchard	P21	ST4	NI
		P1	ST5	NI

[*1]Discarded from the main analysis. NI: not identified; ST: subtype.

Finally, although out of the scope of this study, it is worth mentioning the identification of several genera/species of fungi (Opisthokonta, Nucletmycea), mainly within sardinellas (**Figure 18**), which are recognized as true or opportunistic human pathogens: *Malassezia* spp. and *Wallemia sebis* (Basidiomycota); *Alternaria* sp., *Aspergillus* spp., *Candida tropicalis*, *Cladosporium* sp., *Diutinia rugosa*, *Galactomyces candidus*, *Hortaea werneckii*, *Nakaseomyces nivariensis*, *Nigrospora oryzae*, *Penicillium* sp., and *Sarocladium* sp. (Ascomycota).

4. Discussion

4.1. Blocking primer effectivity

We selected for the purpose of this study a metabarcoding approach designed by Homma et al. (2022) and originally intended for the dietary analysis of herbivorous fish that targets the variable V8-V9 region of the 18S rRNA gene and includes a fish-specific blocking primer. The variable region V9 of the 18S rRNA, together with the V4 region, is one of the most widely regions used for assessing eukaryotic diversity in metabarcoding assays. The V9 region is more advantageous in revealing the eukaryotic diversity, including rare taxa, and has been proven to be effective for studies on parasites (Hino et al., 2016). On the other hand, the V4 region is preferred for inferring evolutionary relationships (Choi and Park, 2020). In addition to the appropriate choice of the primer set to be used, it is advisable to include a blocking primer in the reaction, to avoid host DNA amplification, which can prevent the detection of less abundant eukaryotic taxa (Pompanon et al., 2012). Homma et al. (2022) designed the blocking primer BlockFish_long6 based on several herbivorous fish species. Therefore, we first tested the efficacy of this protocol for our targeted species, which are considered planktivorous feeders (Bachiller et al., 2021). Our results showed that the blocking primer BlockFish_long6 is effective in suppressing the host DNA amplification of both pilchards and sardinellas at a high rate, suggesting that this region is conserved among all Teleostei.

4.2. Parasite community characterization

We detected parasites in all surveyed pilchards and in most of the sardinellas (85%). Pilchards showed a greater presence of all parasitic classes detected, although only significant for Cestoda and Myxozoa. Along with the occurrence, we present data on parasitic abundance, which refers to the proportion of parasitic reads. This must be interpreted cautiously, as the number of parasitic reads does not necessarily reflect the parasitic load. In general, data from sequencing studies should be considered semiquantitative rather than quantitative, as several biological and

technical factors can influence the resulting proportion of each sequence (Pompanon et al., 2012). Among biological factors, the size, the species, and the developmental stage determine the amount of targeted DNA (Prokopowich et al., 2003). Technical factors arise at each processing step: differences in the success of DNA extraction according to the parasitic species or developmental stage (e.g., some resistance forms, like cysts or spores, may require specific extraction protocols); minor differences among taxa in amplicon length or primer binding may bias amplification efficiency; even the sequencing technique and bioinformatic pipelines can affect the resulting proportions (Deagle et al., 2013, Fouhy et al., 2016). Therefore, parasitic abundance can be cautiously useful for comparison between host species within the same metabarcoding assay. As we stated in the results section, pilchards showed significantly higher general parasitic abundance than sardinellas, as well as for the relative abundance of Myxozoa and Cestoda classes. However, comparisons between parasitic organisms should be avoided.

Parasites pertaining to classes Trematoda, Conoidasida, and Myxozoa have been detected in both fish species, while Cestoda was only present in pilchards. Although it was possible to identify as far as eight specific genera (and six species), there were other OTUs that could only be assigned to a higher taxonomic level: class Trematoda, order Azygiida, and family Hemiuridae (in Trematoda); subfamily Eimeriorina (in Conoidasida); order Bivalvulida (in Myxozoa); and Cestoda (**Figure 20**). The impossibility of reaching genus or species levels is quite common when targeting the 18S rRNA gene in metabarcoding assays. Traditionally, 18S rRNA metabarcoding has been used for assessing eukaryotic diversity due to the relative ease of designing universal primers. However, its low evolution rate leads to insufficient variability in the amplified regions for the purpose of distinguishing between closely related organisms. The most common alternative is the cytochrome c oxidase subunit 1 (*COI*) gene, which allows for greater taxonomic deepeness due to its greater variability, but in return, it is more difficult to design broad coverage primers, being sometimes necessary taxon-specific primers

amplifying short regions (minibarcodes) (Tang et al., 2012; Günther et al., 2018). Moreover, the incompleteness of eukaryotic databases also can hamper the identification of some organisms (Cristescu and Hebert, 2018).

Among trematodes, *Aphanurus* was identified in both pilchards and sardinellas, whereas *Didymozoon* and *Maccallumtrema xiphiados* were exclusively detected in sardinellas. Two species, *Aphanurus stossichii* and *Aphanurus virgula*, have been previously reported in pilchards (Marzoug et al., 2012), with only *A. stossichii* described in sardinellas (Feki et al., 2016). However, it is important to highlight that *Aphanurus mugilus* is presently the sole species within the genus from which molecular data are available, relying the other species identification on morphology (Atopkin et al., 2017). *Dydimozoon* (Didymozoidae) is a genus parasitising the gills, mainly in scombrids (e.g., Mele et al., 2014), and *M. xiphiados* (Didymozoidae) has been described from the muscle of the Atlantic swordfish (Ramilo et al., 2023). Therefore the presence of both in the gastrointestinal tract of sardinellas is likely indicative of spureous parasitism. Parasites could potentially be ingested from the contaminated water or via intermediate hosts that are part of the fish diet.

Similarly, the species identified within Myxozoa are not typically infecting the digestive tract of their hosts. *Ceratomyxa* species are known to infect the gallbladder of their hosts (e.g., Kalatzis et al., 2013), while *Sphaerospora dicentrarchi* is a systemic parasite affecting connective tissue (Muñoz et al., 2000), and *Kudoa thyrsites* infects the muscle (Giulietti et al., 2022). Once again, we argue that these findings correspond to spurious parasitism, particularly interesting in the case of sardinellas. The three identified species in sardinellas, namely *Ceratomyxa diplodae*, *Ceratomyxa sargus* n.sp., and *S. dicentrarchi* are common parasites from cultured and wild sparids (gilthead seabream and breams), as well as from European seabass (Muñoz et al., 2000; Rocha et al., 2016; Rocha et al., 2023). All these fish species are either cultivated in offshore fattening farms or are part of the wild synanthropic populations from which the analyzed sardinellas originate. Among these species, *Kudoa thyrsites* stands out for its public health relevance. There are reports pointing

out the allergenic potential of the genus *Kudoa* (Martínez de Velasco et al., 2007; Martínez de Velasco et al., 2008). Moreover, the related species *Kudoa septempunctata* from the olive flounder (*Paralichthys olivaceus*) is recognized as a cause of foodborne illness in Japan (Tachibana and Watari, 2020).

In Conoidasida, *Goussia ameliae* and *Cryptosporidium* sp. were detected only in pilchards. By contrast, in sardinellas even if we detected the genus *Goussia*, it was not possible to reach the species level. To date, *Goussia clupearum* is the single described species affecting both pilchards and sardinellas (Saraiva et al., 2023). Curiously, *G. ameliae* documented here was described from an anadromous fish from the east coast of North America that belongs to the same family as pilchards, Cupleidae (*Alosa pseudoharengus*; Lovy and Friend, 2015). On the other hand, *Cryptosporidium* comprises several species, including zoonotic (Ryan et al., 2014) and fish-specific species (Golomazou et al., 2021). As the metabarcoding assay was only able to reach the genus level, their presence should be considered as potentially zoonotic.

We underline the absence of parasitic microsporidia and nematodes in any of the samples analyzed. Unfortunately, the primer set used does not cover the phylum Microsporidia and shows a very low coverage for nematodes, completely excluding the superfamily Ascaridoidea (*in silico* test in the SILVA database), which includes the main fish nematodes. Among the nematodes, *Anisakis* spp. and *Hysterothylacium* spp. have been frequently reported in pilchards and, to a lesser extent, in sardinellas (e.g., Piras et al., 2014; Fuentes et al., 2022). With regard to microsporidia, the genus *Glugea* has been reported in both fish species (Ramdani et al., 2022). Therefore, even if the absence of these parasites in the analyzed samples could reflect a true absence, it is more likely to be due to false negatives inherent in the technique used.

4.3. PCR targeted detection vs metabarcoding assay

Targeted detection by PCR methods in pilchards showed a very low prevalence for both analyzed parasites, *Cryptosporidium* spp. (2/216; 0.93%) and *Blastocystis* sp.

(2/216; 0.93%). This prevalence is in accordance with the general prevalence obtained for both parasites in wild fish from extractive fisheries in the study in Chapter 1 (0.9% for *Cryptosporidium* and 0% for *Blastocystis*). A previous study reporting *Cryptosporidium* presence in European pilchards, also obtained a similar prevalence (1.3%; Certad et al., 2019), while no previous data for *Blastocystis* sp. in this fish species exist. Conversely, presence in sardinellas seems to be higher for both *Cryptosporidium* spp. (4/25; 16%) and *Blastocystis* sp. (2/25; 8%). However, it must be taken into account the small sample size evaluated for the sardinellas and that they came from the surroundings of the offshore fattening farms (synanthropic group), implying different environmental conditions.

The metabarcoding assay was able to detect the genus *Cryptosporidium* in both positive pilchards and in two further specimens but failed to detect any of the four positive sardinellas. Moreover, it was not possible to achieve species level in any case. It has been noted that taxonomy affinity can fluctuate depending on the primer set (Choi and Park, 2020), masking the detection of some taxons in favor of others with more affinity for the primer set. Therefore, the different overall eukaryotic composition between sardinellas and pilchards could explain the detection of *Cryptosporidium* only in the latter. As mentioned before, the impossibility of reaching species level, even genus, is quite common when using 18S rRNA primers, as the amplified region does not present enough variability to distinguish closely related organisms.

Similarly, the metabarcoding assay failed in detecting *Blastocystis* sp. positive specimens from both fish species, although this genus (*Blastocystis* sp. and *Blastocystis pythoni*) was detected in a very low number of reads in another two sardinellas.

4.4. Advantages, limitations, and future proposals

The metabarcoding approach employed here in the study of the marine fish "parasitome" provided a general map of the parasite diversity with less effort and

cost than traditional techniques based on morphology or PCR. Furthermore, we demonstrated the possibility of characterizing the gastrointestinal parasitic community of marine fish from a metabarcoding assay intended for diet characterization. Dietary studies on aquatic organisms based on a metabarcoding approach have notably increased in the last decade due to the high importance of trophic webs in ecosystem functioning; around half of them targeting the 18S rRNA gene (de Sousa et al., 2019). Therefore, opening the possibility of studying parasitic organisms from already existing databases. Moreover, although out of the scope of this study, we have shown the possibility of unraveling not only host parasites but also parasites from prey (**Supplementary table 4**). Predation of infected prey by hosts unsuitable for the parasite results in parasite consumption rather than transmission. This process is known as concomitant predation and has emerged as an important factor influencing food web structure and dynamics, as well as regulating parasite transmission (Johnson et al., 2010). Therefore, this information could contribute to more comprehensive dietary studies.

Despite the advantages, metabarcoding approaches are not exempt from limitations. The primer set employed in the present study, although considered universal for eukaryotic taxa (Bradley et al., 2016; Homma et al., 2022), has shown very low coverage for an important parasitic taxa (i.e., Nematoda). Even if primers used here target the region V8-V9, they were selected (forward) and designed (reverse) to be adjacent to the conservated fish region where the blocking primer binds to and for the purpose of dietary studies (Homma et al., 2022). Therefore, although these authors took into account all major eukaryotic supergroups, some parasitic taxons within subdivisions probably were not fully assessed. In addition, as discussed before, primers with different affinities could hinder the detection of rare or non-abundant, but interesting species (e.g., zoonotic *Blastocystis* sp. or *Cryptosporidium* spp.). Another important limitation already highlighted, is the low resolution of the taxonomic assignment obtained, mainly inherent to the selected gene, but also because of incomplete databases (Tang et al., 2012; Cristescu and Hebert, 2018).

To at least partially overcome these limitations, we propose the following strategy for future studies: 1) to design blocking primers for other primer sets with better parasite coverage (Kounosu et al., 2019); (2) to apply hierarchical metabarcoding, i.e., to use universal 18S rRNA primers as a first exploratory technique followed by specific taxon metabarcoding based on *COI* gene (Moszczynska et al., 2009), to achieve species taxonomic resolution within the main interest groups, for example, those including zoonotic species.

Recently, UW-Madison researchers have patented a new universal method for parasite identification based on metabarcoding, but using CRISPR-Cas9 digestion with host-specific guide RNAs for reducing host DNA (Goldberg and Owens, 2023), which also presents as a promising strategy.

CHAPTER 3

CHAPTER 3. Contributions to the *in vitro* life cycle of the zoonotic nematode *Anisakis pegreffii*: fecundity, *in vitro* early larval development, and karyotype

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This chapter is in press in the Veterinary Parasitology journal:

1. Moratal, S., Zrzavá, M., Hrabar, J., Dea-Ayuela, M.A., López-Ramon, J., Mladineo, I. (in press). Fecundity, *in vitro* early larval development and karyotype of the zoonotic nematode *Anisakis pegreffii*. Vet. Parasitol.

1. Introduction

Research of fundamental mechanisms of helminth function, survival, and reproduction has been incentivized by the pioneering use of -omics tools, enabling major steps in translating existing knowledge in measures to mitigate and control of highly prevalent neglected diseases (International Helminth Genomes Consortium, 2019; Jasmer et al., 2019). While relying on environmental and clinical samples of helminths as a source for downstream -omics protocols is practical and fitting for the variety of experimental hypotheses, other protocols may require higher numbers of developmental stages that are not always available in nature, or clonal and less heterogeneous parasite populations grown under specific conditions, especially in cases where gene functions are explored through gene editing techniques (Gang et al., 2020; Cheong et al., 2021). Therefore, *in vitro* life cycle protocols for helminths, especially zoonotic taxa, represent a fundamental tool providing ideal conditions for rearing parasites in large numbers, repeatability, and a reliable environment for comparing mechanisms in wild-type specimens. However, even the number of known natural life cycles of trophically transmitted helminths is considerably small (i.e., 973) relative to the number of host-parasite-species associations (Benesh et al., 2017). This results in knowledge gaps that prevent us from fully understanding helminth co-evolution and infection and evasion strategies - important variables to consider in parasitosis management.

Anisakiosis is an emerging zoonosis caused by the sibling species *Anisakis simplex* sensu stricto (s.s.) and *Anisakis pegreffii* (Anisakidae) (Adroher-Auroux and Benítez-Rodríguez, 2020) contracted through the consumption of raw or inadequately processed fish infected with third-stage nematode larvae (L3). The disease is usually associated with an acute gastrointestinal or gastro-allergic reaction (Daschner and Pascual, 2005; Audicana and Kennedy, 2008), which has been known for a long time (i.e., the first observation was reported in 1876 (Leuckart, 1876)) but remains misdiagnosed, under-reported, and unknown in countries with below-average fish consumption. In contrast, reports suggest that the number of cases is increasing in

proportion to changes in food consumption habits worldwide (EFSA, 2010; Llarena-Reino et al., 2015), resulting in anisakiosis ranking fifth in the European risk classification and being the second of 24 foodborne parasitoses with the highest increasing illness potential (Bouwknegt et al., 2018).

Recently, a reliable protocol for *in vitro* culture of *A. pegreffii* has been established, allowing adult development, harvest of fertilized eggs and hatching of second-stage larvae (L2) (Mladineo et al., 2023). However, no further development towards the later larval or adult stages was followed in the study, which would help to elucidate certain aspects of the biology of the species. In the marine environment, hatched and free-swimming L2 are preyed upon by crustaceans (mainly euphausiids) and possibly small fish (Aibinu et al., 2019). L2 are thought to grow in the crustacean haemocoel after ingestion and moult into L3 (Smith, 1983), although the specific conditions and cues required for this process are as yet unknown. Of particular interest is the development of the excretory gland cell (EGC). This conspicuous cell is considered the main production site for the excretory/secretory products (ESPs) and extracellular vesicles (EVs) of anisakids (Cavallero et al., 2018; Mladineo et al., 2023) and is thus a *conditio sine qua non* for efficient virulence and propagation of the nematode. ESPs and EVs secreted by L3 contribute to the activation of kinases involved in inflammation and cell proliferation (or inhibition of apoptosis) (Messina et al., 2016), as well as to processes supporting inflammation, apoptosis, cell proliferation, and differentiation, respectively (Cavallero et al., 2022b). Thus, the ontogeny of EGC through earlier stages could provide valuable insights into the host-parasite interaction of anisakids, which is particularly interesting from a co-evolutionary aspect of a parasite inhabiting multiple hosts.

The main objective of this study was to test the conditions for *in vitro* development of L3 stages from *A. pegreffii* eggs and to determine the morphological characteristics of early L2 larvae using transmission electron microscopy (TEM). Furthermore, capitalising on two adult populations maintained *in vitro*, i.e., *A. pegreffii* from the Adriatic Sea and a mixed *A. simplex* sensu lato (s.l.) population from the east coast of

the North Atlantic, we monitored and compared fecundity over time, thus obtaining the first long-term data on the fecundity of anisakids *in vitro*. Finally, we obtained the first chromosome spreads of *A. pegreffii* and defined the karyotype of the species, which may be a valuable tool for molecular cytological studies in the future and contribute to better genome assembly and chromosome mapping (Doyle, 2022).

2. Materials and methods

2.1. Origin of *Anisakis* spp. L3

Anisakis spp. type I larvae were collected from naturally infected blue whiting *Micromesistius poutassou* acquired from commercial fishermen in two different locations: Adriatic Sea (Croatia, FAO Division 37.2.1) and North Atlantic (Spain, FAO Division 27.8). Fish were stored on ice until dissection at the Institute of Oceanography and Fisheries, Croatia (Adriatic population) and at the Faculty of Health Sciences from University Cardenal Herrera-CEU, Spain (North Atlantic population). The actively moving L3 were removed from the viscera and visceral cavity with small forceps, washed with physiological saline, placed in 50 mL tubes containing autoclaved seawater and shipped to the Institute of Parasitology in the Czech Republic. Immediately after arrival, larvae were washed in autoclaved M9 buffer containing 2% penicillin/streptomycin (PS) (Sigma, P4333-20ML) and amphotericin B (Sigma, A2942-20ML) (three 30-min washes at room temperature and protected from light). Finally, larvae were examined under a stereomicroscope (Zeiss, Stemi 305) for morphological genus confirmation and integrity.

2.2. Adults *in vitro* culture and eggs harvesting

L3 larvae were cultured in autoclaved glass bottles (one batch for the Adriatic population and one batch for the North Atlantic population) to reach the adult stage, according to the protocol recently established by Mladineo et al. (2023).

After observing the first eggs expelled in the medium in the 3rd week in culture, the eggs were collected for subsequent quantification each time the medium was

changed (three times per week). This means that the eggs expelled were produced over 48 h (if collected on Wednesdays and Fridays) or 72 h (if collected on Mondays). The medium was collected in 50 mL tubes and centrifuged at 22x*g* for 20 min at 19 ºC. After discarding the supernatant, the pellet was washed twice in 5 mL of autoclaved sea salt solution (SSS) [31.73 g of Sea salts NutriSelect Basic (Sigma, S9883-500G) in 1 L of distilled water] by centrifugation under the previous conditions. The eggs were resuspended in autoclaved SSS and filtered through a 70 µm pore size nylon cell strainer (Corning, 734-2761). After another centrifugation cycle, the supernatant was discarded and eggs were resuspended in 1 to 3 mL of SSS, depending on the number of eggs (more SSS for a larger egg pellet). The resuspension was thoroughly mixed by pipetting and used to fill a Bürker chamber. Quantification was performed under the light microscope (Olympus, CKX53) according to the standard protocol for blood cell counting in the Bürker chamber:

Eggs/mL = (Total eggs counted x 10,000 eggs/mL)/Number of squares counted
Total eggs/medium = Total eggs/mL x Volume of sample containing the eggs

In addition, the number and sex of the dead adult specimens were noted at each egg harvesting.

Eggs for L2 culture were collected following the same protocol with few modifications. After changing the medium on a given day, the adults were left in the new medium for a maximum of 4 h. Eggs were then collected to ensure that only recently expelled eggs were harvested and to minimize the potentially deleterious effect of the acidic culture medium on the eggs. Eggs were centrifuged and washed twice in complete SSS; autoclaved SSS containing 2% PS and nystatin (1 mL/L) (Sigma, N9150-20ML). The pellet was resuspended in 1-3 mL of complete SSS, passed through a cell strainer to remove cell debris, and the filtrate was divided into 50-100 µL aliquots in 6 well plates. A further 2 mL of complete SSS were added and the eggs were incubated at 19 ºC in a 5% CO_2 atmosphere. Under these conditions, the first L2 hatched on day five (6-7 days for the early egg batches) and were left for

a further 48 h due to asynchronous hatching. 48 h after hatching, the larvae were separated from the unfertilized and unhatched eggs with a 30 μm mesh cell strainer (pluriStrainer® S/ 30μm, pluriSelect) and collected in a Petri dish for subsequent culture testing.

2.3. Fecundity

The fecundity was expressed as the number of eggs expelled per day in *A. pegreffii* and *A. simplex* s.l. colonies. At the same time, we recorded the number, sex, and date of adults removed from the culture (due to other sampling requirements and mortality). Using the total number of adults initially placed in the culture and the calculated sex ratio (expressed as a percentage of females) at each count, we plotted the number of eggs per day and per reproductive female. Day 0 corresponds to the day when the L3 were placed in culture to obtain the adults. Fertility (here considered as the percentage of hatched L2 out of a total number of eggs collected) was not assessed quantitatively, but we recorded the time point at which fertilization of eggs started and stopped.

2.4. Culture of *A. pegreffii* L2, survival and exsheathment rates

Culture of *A. pegreffii* L2 obtained *in vitro* was performed in commercial Schneider's *Drosophila* Medium (SDM) (Gibco, 21720024) supplemented with 1% sodium pyruvate (Sigma, S8636-100ML), 5 μg/mL liver concentrate (Sigma, 2023-50G), 10% chicken serum (CS) (Merck, C5405-100ML), 2% PS and 1 mL/L nystatin. The complete medium (pH ≈7 and osmolarity ≈300 mOsm) was syringe filtered (0.22 μm, Techno Plastic Products, 99722) before use.

By manual micropipetting (100 μL tips), 10 harvested L2 no older than 48 h were redistributed into a 24-well plate containing SDM (19 ºC; 500 μL/well) prepared at different osmolarities (300, 400, 500, 600, 700, 800 mOsm) and in SSS + SDM (1:1), each in duplicate. The change in osmolarity was adjusted by adding 5 M NaCl directly to the wells containing the medium and gently stirring the plate. Larvae in

SSS were considered as controls. The plate containing L2 was incubated at 19 ºC in a 5% CO_2 atmosphere.

Preliminary tests showed that although L2 grew in Dulbecco's modified Eagle's medium-low glucose and -high glucose and in SDM for at least two weeks, survival was higher in the latter, as was L2 size, when 20-50% CS was added (Mladineo et al., 2023). We selected lower CS concentration (10%) to allow balanced growth and stable exsheathing processes. Based on the observation that crustaceans and thus anisakids do not thrive in low salinity environments (e.g., brackish water), different osmolarities were tested. However, the osmolarity values in the haemocoel of crustaceans where L2 moults into L3 are also variable and depend on the crustacean species, moulting cycle, body weight or nutritional status (Van Mai and Fotedar, 2018).

The larvae were checked daily under the microscope (Olympus, CKX53) and the medium was partially replaced once a week by surface aspiration. The experiment was terminated at week 11 when less than 15% of the larvae were still alive. The remaining larvae were pooled in two wells with culture medium at 700 mOsm: one well contained the "small L3 phenotype" and the other the "large L3 phenotype". These larvae were kept in culture for further microscopy, the last four surviving until week 17.

Larval survival, number of exsheathed larvae, and observation of large L3 phenotypes were recorded three times per week and plotted in ggplot2 package (Wickham, 2016) for R (R version 4.1.2; R Core Team, 2021). To test the effect of osmolarity on survival and exsheathment frequency, pairwise comparisons were performed using Fisher's exact test with Benjamini-Hochberg FDR correction method using R software (R core Team, 2021) and rstatix package (Kassambara, 2023). Significance was set at a *p*-value of less than 0.05.

2.5. Morphological characterization of *A. pegreffii* early developmental stages

2.5.1. Morphology and morphometry

Samples of recently expelled eggs (<24 h; N=35), embryonated eggs before hatching (6 days incubation, N=30), recently hatched larvae (<48 h, N=25), un-exsheathed larvae cultured for 3 weeks (N=16), and exsheathed larvae (N=7) were either fixed with paraformaldehyde 4% or left in SSS to be examined under contrast phase microscope (Olympus BX63F with camera DP74). Measurements were performed using cellSens Dimension Desktop 2.2 with the CI Deconvolution package and images were assembled and annotated in Inkscape 1.0 software (https://inkscape.org).

2.5.2. Transmission electron microscopy (TEM)

Recently expelled eggs (<24 h), embryonated eggs before hatching (6 days incubation), and recently hatched larvae (<48 h) were used for TEM study. Eggs and larvae were transferred to 1.5 mL tubes in autoclaved SSS and centrifuged at 60x*g* for 5 min. The supernatant was discarded and the pellet containing the eggs/larvae was transferred to 0.2 mL PCR microtubes. After centrifugation (5 min, 60x*g*), the eggs/larvae were collected from the pellet using cellulose capillary tubes with an inner diameter of 200 μm, fibre length of 50 mm, and wall thickness of 8 μm (LEICA, 16706869), which were immediately processed for high-pressure freezing and freeze substitution as described in Mladineo et al. (2023). Briefly, samples were washed in acetone (3×15 min each) and infiltrated in 25, 50 and 75% mixtures of low viscosity Spurr resin (SPI Chem, West Chester, PA, USA) and anhydrous acetone for 1 h each, and left overnight in 100% resin. The samples were then placed in embedding moulds for polymerization (48 h at 60 ºC). Semi-thin sections (0.5 μm) stained with toluidine blue (1%) were checked for orientation under the light microscope. Ultrathin sections (0.07 μm) were mounted on Formvar-coated single-slot grids and contrasted in ethanolic uranyl acetate (30 min) and lead citrate (20 min) before observation under a JEOL JEM-1400 microscope (JEOL, Akishima,

Tokyo, Japan) operating at an accelerating voltage of 120 kV. The images were taken with a XAROSA 20-megapixel CMOS camera (EMSIS GmbH) and assembled and annotated in Inkscape 1.0 software (https://inkscape.org).

2.6. *A. pegreffii* karyotype

2.6.1. Chromosome preparations

Ten adult *A. pegreffii* obtained *in vitro* were used to prepare chromosome spreads (5 males and 5 females; 6-11 weeks old). Specimens were dissected in physiological solution (Glaser, 1917) under a stereomicroscope (Zeiss, Stemi 305), and the gonadal tissues were immediately placed in hypotonic solution (0.075 M KCl) for 5 min. Tissues were then transferred to freshly prepared Carnoy's fixative (6:3:1; ethanol - chloroform - acetic acid) for 10-15 min. After fixation, a very small piece of tissue (1-5 mm long) was transferred onto a superfrost slide (previously washed for one hour in acidic ethanol [0.5 mL HCl + 50 mL 99% EtOH]) in a 10 μL drop of 60% acetic acid and torn into fine fragments using two tungsten needles. The slide was then heated on a hot plate at 45 ºC, and the drop of acetic acid was moved with a tungsten needle in an area of less than 24 x 40 mm until the liquid evaporated. Finally, the slide was dehydrated through an ascending ethyl alcohol series (70%, 80%, 99%; 60 s each) and air dried. A Jenalumar phase contrast microscope (Carl Zeiss Jena, Germany) was used to check the quality of the spreads before DAPI (4',6-diamidino-2-phenylindole) staining or fluorescence *in situ* hybridization (FISH).

2.6.2. DAPI staining and FISH with biotin-labelled telomeric probe

Part of the slides were stained directly by adding a drop of Fluoroshield™ with DAPI (Sigma, F6057-20ML) and covered with a coverslip. The rest of the slides were frozen and later used for FISH of nematode telomeric sequence.

The telomeric nematode probe $(TTAGGC)_n$ was synthesized by the non-template PCR method (following Sahara et al., 1999) and labelled with biotin-16-dUTP by nick translation using the Biotin16 NT Labeling Kit (PP -310L-BIO16, Jena

Bioscience) according to the manufacturer's instructions.

We used the FISH procedure, combining hybridization described in Sahara et al. (1999) with the post-hybridization washing and detection procedures described in Cabral-de-Mello and Marec (2021). Briefly, the hybridization mixture contained 50 ng of the biotin-labelled probe, 25 μg of sonicated salmon sperm DNA (Sigma Aldrich, St. Louis, MO, USA), 50% deionized formamide, 10% dextran sulphate, and 2x saline sodium citrate buffer (SSC). Chromosome slides were removed from the freezer, passed through the ethanol series (70%, 80%, and 100%, 30 s each) and air dried. The slides were then denatured in 70% deionized formamide in 2 x SSC for 3 min 30 s at 68 ºC, incubated in cold 70% ethanol (-20 ºC, 1 min), passed through 80% and 100% ethanol and air dried. The hybridization mixture was denatured at 90 ºC for 5 min, immediately cooled on ice and applied to the denatured slide. Hybridization took place overnight at 37 ºC. Washes after hybridization were as follows: twice in 2 × SSC at 42 ºC for 5 min, twice in 0.1 × SSC at 42 ºC for 5 min, once in 2 × SSC for 5 min at 42 ºC, once in 2 × SSC in a Coplin jar for 10 min at room temperature, incubation in WBB (4 × SSC, 0.1% v/v Tween 20, 1% w/v skimmed milk) in a Coplin jar for 15 min at room temperature. Probe detection was performed with streptavidin-Cy3 (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA) in WBB for 1 h at 37 ºC. Then the slide was washed three times in WBB in a Coplin jar for 5 min each at 45 ºC and mounted in DAPI (c = 1 μg/mL) in DABCO antifade.

2.6.3. Microscopy and image processing

DAPI and FISH slides were observed under confocal and fluorescence microscopes (Olympus FV 3000 and Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany), respectively). For FISH slides, black and white images were taken separately for each fluorescent dye and later pseudo-colored (red for Cy3 and light blue for DAPI) and merged using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA).

2.7. Molecular identification

A subsample of 32 adult nematodes from *in vitro* culture was used for molecular identification of *Anisakis* spp., comprising 15 and 17 individuals from the North Atlantic and Adriatic batches, respectively. DNA was extracted using the SSTNE buffer and salt precipitation as previously described (Bartie et al., 2020). A ~600 bp fragment of cytochrome c oxidase subunit 2 (*cox2*) was amplified by combining 50 ng of purified DNA, 12.5 μL Premix Ex Taq Hot Start Version (Takara, Shiga, Japan), 0.5 μL (0.2 mM) of each primer, forward 211F 5′- TTT TCT AGT TAT ATA GAT TGR TTYAT -3′ and reverse 210R 5′- CAC CAA CTC TTA AAA TTA C-3′ (Nadler and Hudspeth, 2000), and nuclease-free water to a volume of 25 μL. Cycling conditions were set as follows: 30 cycles for 10 s at 98 ºC, 30 s at 46 ºC (annealing), and 1 min at 72 ºC. PCR products were visualized in a 1% agarose gel stained with SYBRTM Safe (Invitrogen, Waltham, MA, USA) and commercially sequenced (Macrogen Europe Laboratory, The Netherlands). The sequences obtained were aligned with the sequences of *Anisakis* spp. available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (*A. simplex* s.s. (DQ116426), *A. pegreffii* (DQ116428.1), *Anisakis berlandi* (DQ116429.1), *Anisakis ziphidarum* (DQ116430.1), *Anisakis typica* (DQ116427.1), *Anisakis nascettii* (DQ116431.1), *Anisakis physeteris* (DQ116432.1), *Anisakis paggiae* (DQ116434.1), *Anisakis brevispiculata* (DQ116433.10029)) by the Clustal W algorithm implemented in the Mega X software (Kumar et al., 2018), checked for incorrectly read bases and manually corrected if necessary. Species were identified by comparing the sequences with those available in GenBank using BLASTn (Altschul et al., 1990). Sequences were deposited in GenBank with accession numbers OQ919751-OQ919762 for *A. simplex* and OQ919763-OQ919782 for *A. pegreffii*. Bayesian inference analysis was performed in MrBayes 3.2.7 (Ronquist et al., 2012) to discriminate between specimens of *A. simplex* s.s. and *A. pegreffii* (**Supplementary figure 1**).

The same samples were additionally genotyped using PCR-based restriction fragment length polymorphism (PCR-RFLP) to discerne potential recombinant

(hybrid) genotypes. Samples were amplified as described above with forward primer BD1 5′- GTC GTA ACA AGG TTT CCG TA -3′ and reverse primer BD2 5′- TAT GCT TAA ATT CAG CGG GT -3′ at the locus of the internal transcribed space region (*ITS*) of the rDNA. Cycling conditions were the same as above except that the annealing temperature was set to 56 ºC. Successfully amplified samples were digested with 5U HinfI endonuclease (Promega, Madison, WI, USA) and visualized in 2% agarose gel. Species were identified using the RFLP pattern according to D'Amelio et al. (2000).

3. Results

3.1. Molecular identification

Based on the BLASTn results for the 584 bp long *cox2* sequences, 12 specimens (37.5%) were identified as *A. simplex* (all of them originating from the North Atlantic batch), and 20 specimens were identified as *A. pegreffii* (17 specimens originating from the Adriatic batch and three specimens from the North Atlantic batch), showing 99.14 - 100% and 99.83 - 100% identity with the sequences deposited in GenBank, respectively. The identity of the species was also confirmed by phylogenetic relationship reconstruction. The Bayesian inference consensus tree clustered the 12 identified *A. simplex* specimens with the respective reference sequence, while 20 *A. pegreffii* specimens were clustered with the respective *A. pegreffii* reference sequence (**Supplementary figure 1**).

PCR-RFLP with HinfI revealed three distinct patterns: i) three fragments of approximately 370 bp, 300 bp, and 250 bp corresponding to *A. pegreffii*; ii) two fragments of approximately 620 bp and 250 bp corresponding to *A. simplex*; iii) four fragments of approximately 620 bp, 370 bp, 300 bp, and 250 bp corresponding to the recombinant genotype (putative hybrid). Based on the RFLP pattern, 12 (37.5%) specimens were identified as *A. simplex*, 18 specimens (56.25%) were identified as A. *pegreffii*, and two specimens (6.25%) were assigned recombinant genotypes. Both specimens with recombinant genotypes at the *ITS* locus had *A. pegreffii* matrilineage.

3.2. Fecundity

The batch of *A. pegreffii* consisted of 57 individuals, with the sex rat*io*, i.e., the percentage of females, ranging from 37.50 to 40% (variable at different times due to mortality or sampling of specimens for other protocols). The first eggs were detected on day 21, albeit in very small numbers. **Figure 21** shows the number of eggs per day per female, starting from day 25 until the end of the experiment (day 120). The experiment was terminated due to increased adult mortality and probable fungal contamination from dead specimens, although egg production did not decrease at this time. Average production was 24,370.96 ± 12,564.86 eggs/day/female (mean ± standard deviation), with a minimum and maximum of 1,562.79 and 43,007.75 eggs/day/female, respectively. Fertilized eggs were present from day 31 to day 86, although fertility was not quantitatively assessed. Hatching of embryonated eggs occurred between days 5-7.

Fifty individuals formed *A. simplex* batch, with sex ratio ranging from 51.06 to 69.23% throughout the experiment. Similar to *A. pegreffii*, the first eggs were detected on day 24. The number of eggs per day per female is shown in **Figure 21**, from day 27 until the end of the experiment (day 162). In this case, the experiment was terminated when < 5 worms remained (extractions mainly due to sampling). The average, minimum and maximum egg production was 29,914.05 ± 27,629.36, 713.58 and 115,694.45 eggs/day/female, respectively. Fertilized eggs were present at least between days 64 and 108 and also hatched between days 5-7.

3.3. *A. pegreffii* L2 survival and exsheathment

A. pegreffii L2 showed a high survival and growth rate under all conditions tested, except for larvae in SSS (control) where no growth was observed, although the majority remained alive and active until the middle of the sixth week. Larvae in the highest osmolarities (700 and 800 mOsm) and in the mixture of SSS and DSM (1:1) had the highest survival rate throughout the experiment (**Figure 22**). Significant differences in survival rates for the different conditions tested were observed

between the 6th and 10th week of the experiment. However, the higher survival rates (700 mOsm, 800 mOsm, and the SSS + DSM mixture) did not show a significant difference between them at any time-point during the experiment (**Table 10**).

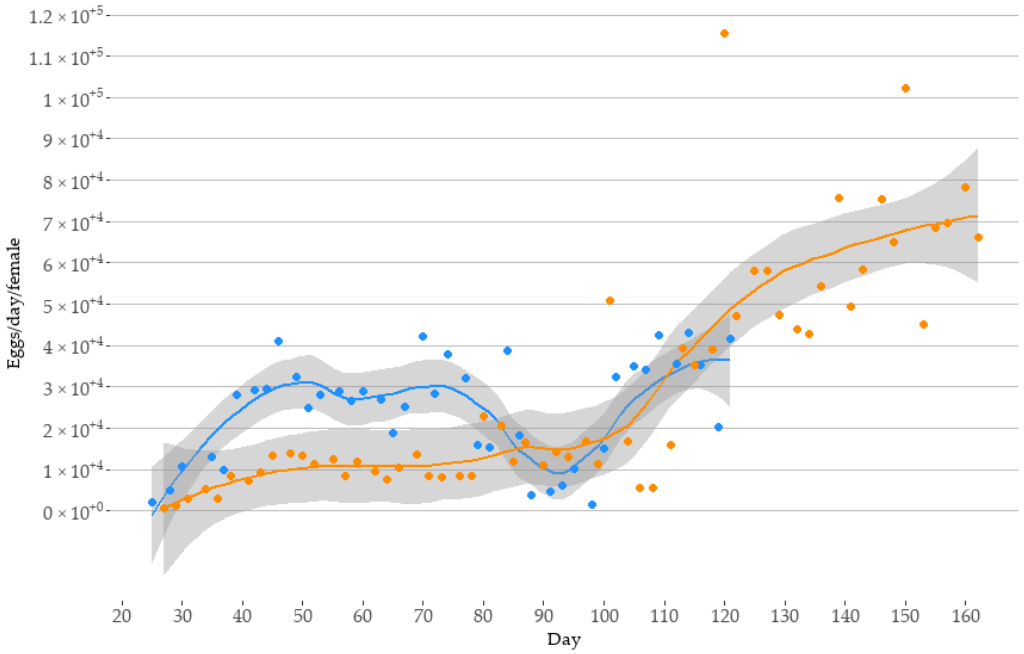

Figure 21. Number of eggs shed per day and female for batch of *Anisakis pegreffii* (blue) and *Anisakis simplex* sensu lato (orange) cultured *in vitro*.

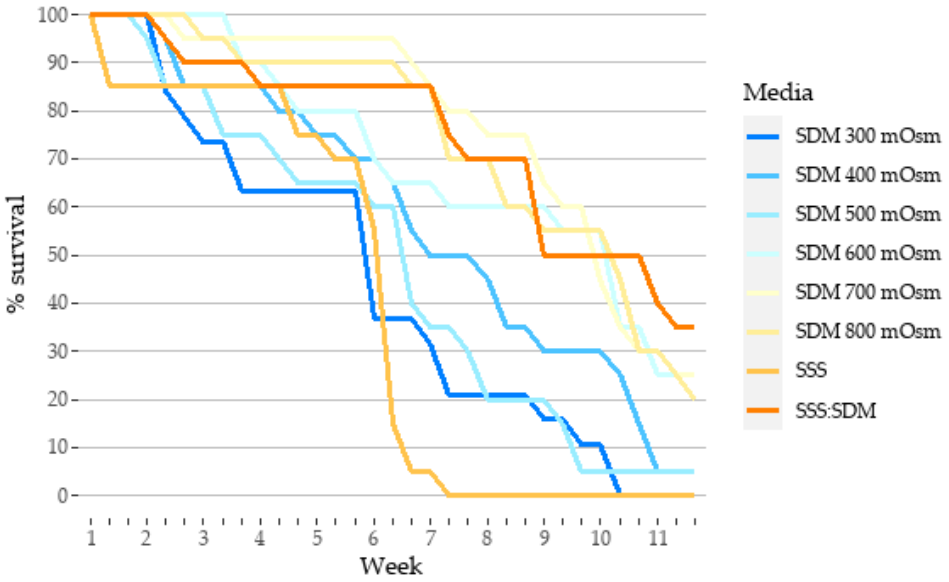

Figure 22. Survival rate of *Anisakis pegreffii* larvae cultured *in vitro* under different osmolarities.

Detachment of the outer cuticle (L2) began at the end of the third week for larvae in 300, 400, and 600 mOsm and during the fourth week for larvae in SSS + DSM (1:1), 500, 700, and 800 mOsm. Interestingly, the control larvae in SSS did not shed the outer cuticle, except for a single specimen. The percentage of exsheathed larvae remained low under all conditions tested until the beginning of the sixth week. One week later (beginning of the seventh week), 100% of the remaining larvae had shed the cuticle (**Figure 23**) and these were considered third stage (L3) larvae. Significant differences in moulting rate were only observed at the midpoint of the experiment (week 6), which coincided with the peak of moulting. Differences were observed between the control (SSS) and the other conditions (except for the SSS + DSM mixture) and between the SSS + DSM mixture and the higher osmolarities (500-800 mOsm) (**Table 10**).

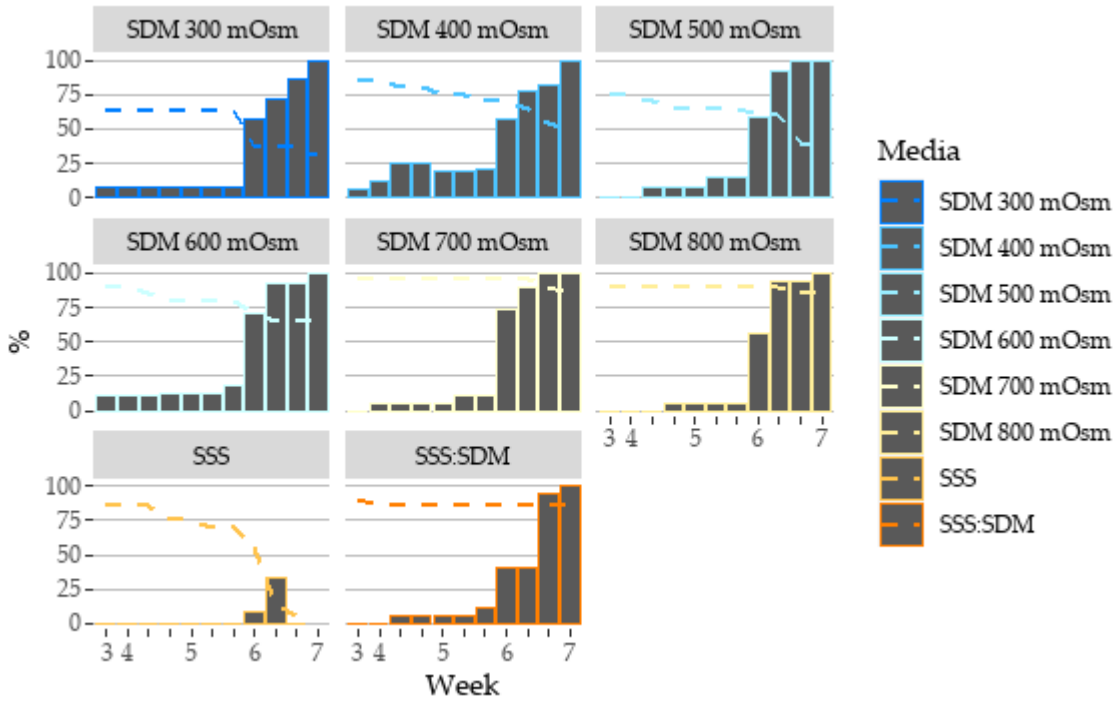

Figure 23. Survival rate of *Anisakis pegreffii* larvae (dashed-line) and percentage of exsheathed larvae (bars) cultured *in vitro* under different osmolarities (independent graphs). The graph encompasses the period between the first exsheathment until 100% of larvae were exsheathed (x axis).

Table 10. Results of pairwise comparisons for survival and exsheathment rates of *Anisakis pegreffii* cultured in Schneider's *Drosophila* medium adjusted to different osmolarities (Fisher's exact test with Benjamini-Hochberg FDR correction). Only significant values ($p < 0.05$) are shown.

	Survival[a]					Exsheathment[b]	
	W6	**W7**	**W8**	**W9**	**W10**	**W6-D1**	**W6-D3**
SSS-SSS:SDM		$3.79x10^{06}$	$3.12x10^{05}$	$3.05x10^{03}$	$4.07x10^{03}$		
SSS-SDM300							$3.58x10^{02}$
SSS-SDM400		$8.50x10^{03}$	$4.92x10^{03}$		$4.71x10^{02}$		$6.30x10^{03}$
SSS-SDM500							$7.49x10^{04}$
SSS-SDM600		$9.59x10^{04}$	$3.16x10^{04}$	$6.31x10^{04}$	$2.03x10^{03}$		$7.49x10^{04}$
SSS-SDM700		$3.79x10^{06}$	$2.16x10^{05}$	$3.61x10^{04}$	$5.83x10^{03}$	$4.76x10^{02}$	$3.08x10^{04}$
SSS-SDM800		$3.79x10^{06}$	$3.12x10^{05}$	$1.35x10^{03}$	$2.03x10^{03}$		$1.61x10^{04}$
SDM300-SSS:SDM	$2.86x10^{02}$	$4.24x10^{03}$	$9.27x10^{03}$		$3.51x10^{02}$		
SDM300-SDM400							
SDM300-SDM500							
SDM300-SDM600			$4.85x10^{02}$	$3.36x10^{02}$	$1.79x10^{02}$		
SDM300-SDM700	$3.84x10^{03}$	$4.24x10^{03}$	$4.92x10^{03}$	$1.71x10^{02}$			
SDM300-SDM800	$1.08x10^{02}$	$4.24x10^{03}$	$9.27x10^{03}$		$1.79x10^{02}$		
SDM400-SSS:SDM							
SDM400-SDM500							
SDM400-SDM600							
SDM400-SDM700							
SDM400-SDM800							
SDM500-SSS:SDM		$8.50x10^{03}$	$9.27x10^{03}$		$1.34x10^{02}$		$2.49x10^{02}$
SDM500-SDM600			$4.85x10^{02}$		$5.83x10^{03}$		
SDM500-SDM700		$8.50x10^{03}$	$4.92x10^{03}$	$3.81x10^{02}$	$2.34x10^{02}$		
SDM500-SDM800		$8.50x10^{03}$	$9.27x10^{03}$		$5.83x10^{03}$		
SDM600-SSS:SDM							$2.36x10^{02}$
SDM600-SDM700							
SDM600-SDM800							
SDM700-SSS:SDM							$1.57x10^{02}$
SDM700-SDM800							
SDM800-SSS:SDM							$5.29x10^{03}$

[a]Statistical differences in survival rate were assessed on counts corresponding to the first day of each week. [b]Statiscal differences in exsheathment rate were assessed for all counts during the weeks of the moulting process. Shades of grey represent a heatmap from the lowest to the highest significance values. D: day; SDM: Schneider's Drosophila medium; SSS: Sea Salt Solution; W: week. Note that the numbers ranging from 300 to 800 in the acronym of medium represent tested values of osmolarities.

L2 showed rapid and flicking movements before exsheathment, accumulated detritus on the outer cuticular sheath and few entangled L2 were usually observed. As the larvae grew larger and went through the exsheathment process, their activity decreased, became more coiled (worm-like), and less detritus was observed attached to the L3 cuticle. During the exsheathment process, the larvae also showed constrictions due to cuticular rings (small remnants of the unshed cuticle), mostly in the anterior or posterior third of the body (**Figure 24A**). Some L3 were able to overcome this, while some reached the "large L3 phenotype" with constrictions still visible. Most larvae managed to shed the cuticular ring (**Figure 24B**). However, in some cases it was observed that the moulting process failed, and the constriction affected the development of the internal organs, leading to the death of the larva. Another moult of the developed L3 was observed after 3 months in culture, occasionally leaving cuticle at the tip of the tail.

Figure 24. A) Exsheathing larva of *Anisakis pegreffii* under contrast phase microscopy. The arrow points to the constriction caused by the cuticle of the second-stage larva (L2) and the arrowhead to the cuticle leftover. B) Recently shed striated L2 cuticle under light microscopy. Scale bars = 50 μm.

3.4. Morphological characterization of *A. pegreffii* early developmental stages

3.4.1. Morphology and morphometry

Morphometric measurements showed that the eggs increased in size during embryonic development while still maintaining a slightly ellipsoidal morphology

(**Table 11)**. The eggs and the recently hatched, double-ensheathed L2 exhibited morphological characteristics consistent with those previously observed (Mladineo et al., 2023) (**Figure 25A-C**).

Table 11. Morphometric measurements of early stages of *Anisakis pegreffii* cultured in Schneider's *Drosophila* medium (mean ± standard deviation; μm) recorded from each developmental stage.

Eggs					
	N	**Long axis**		**Short axis**	
<24 h	37	53.84 ± 4.03		48.18 ± 2.83	
Embryonated (5 d)	29	60.90 ± 4.95		56.03 ± 3.64	
Larvae					
	N	**Body[a]**		**Outer sheath**	
		Length	**Width**	**Length**	**Width**
L2 - <48 h	25	261.81 ± 29.73	22.06 ± 2.48	378.11 ± 20.69	28.99 ±2.28
L2 - 3 weeks	16	338.42 ± 50.40	30.41 ± 7.79	399.87 ± 48.16	32.64 ± 6.90
L3 - 3 weeks	2	466.10 ± 44.60	50.50 ± 1.50	---	---
S3P - 17 weeks	1	463.94 ± 3.99	41.26 ± 0.36	---	---
L3P - 12 weeks	1	1684.70 ± 8.23	72.34 ± 6.23	---	---
L3P - 17 weeks	3	2273.56 ± 522.85	117.79 ± 4.41	---	---

[a]Body measurements of third-stage larvae (L3) were taken per triplicate to reduce the error derived from the use of the manual measuring tool applied for length (sinusoidal shape) and to provide the average of different body widths (proximal, middle, and distal part). Width of second-stage larvae (L2) was taken at the level of mid pharynx. S3P: small L3 phenotype; L3P: large L3 phenotype

The larvae in culture grew rapidly so that the body became tightly enveloped by the outer L2 cuticle, which previously only loosely encased the L2 at hatching. This led to the first exsheathment process of L2, marking the development of L3. L3 continued to grow in length and thicker, showing more developed internal organs. It is worth noting that two phenotypes could be observed during L3 growth, which we termed the "small L3 phenotype" (S3P) and "large L3 phenotype" (L3P), reflecting their significant difference in size. Table 11 summarizes the morphometric data of each developmental stage. As the L3 grew, development continued at a

slower pace, but typical L3 morphological features were observed: developed lips, boring tooth, a pharynx, a ventricle, a developed intestine (with peristaltic movements), a rectum, and an excretory duct (**Figure 25D-G**).

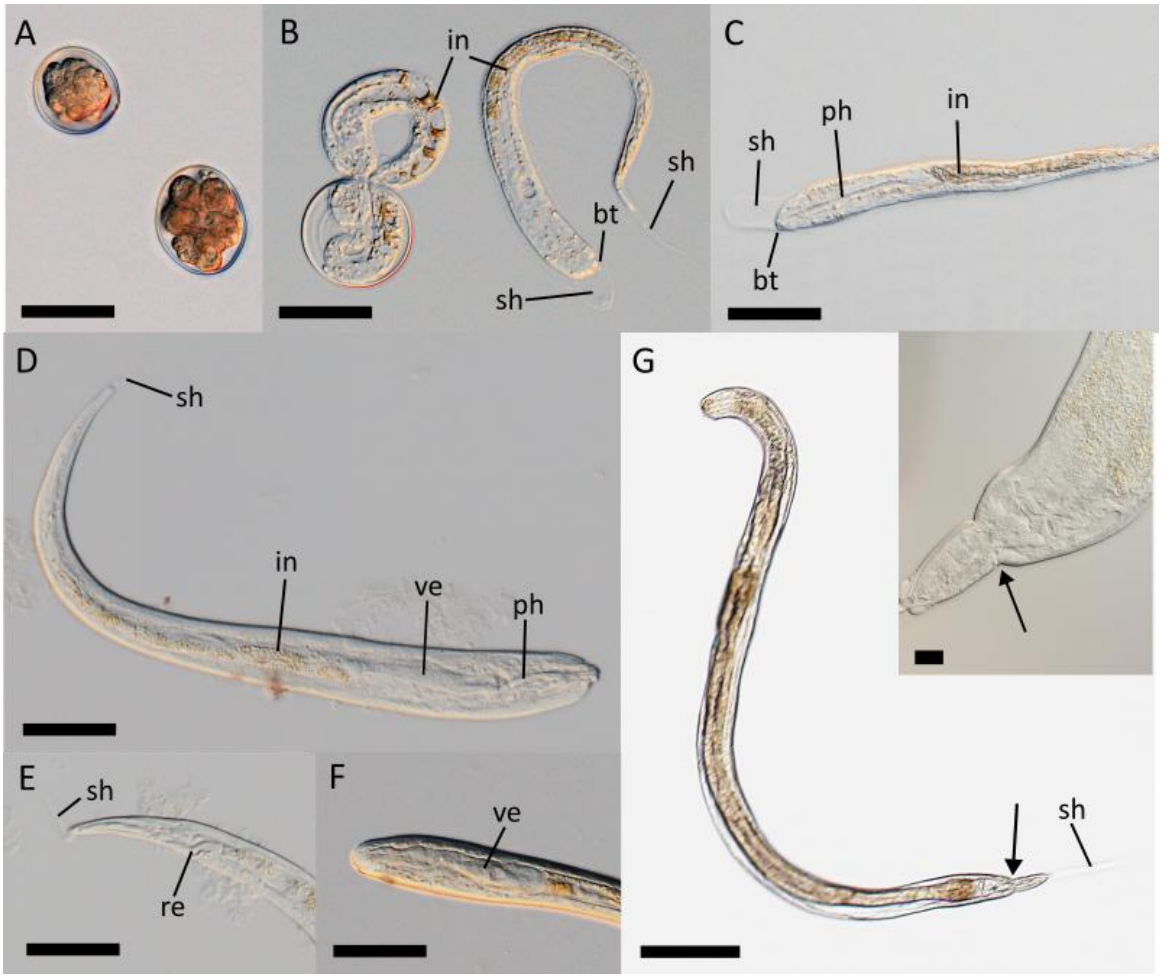

Figure 25. Eggs and larvae of *Anisakis pegreffii* under contrast phase microscopy. A) Eggs at early stages of embryonic development; note the triple-layer, smooth eggshell. B) Larva hatching and recently hatched second-stage larva (L2). C) Double-ensheathed L2. D) Fully developed larva before exsheathment. E) Posterior end of a fully developed larva before exsheathment. F) Anterior part of a fully developed larva before exsheathment. G) Exsheathed third-stage larva (L3) (large L3 phenotype). The inset is the detail of the posterior end, showing the constriction caused by the leftover of the L2 cuticle (arrow). bt: boring tooth; ph: pharynx; in: intestine; sh: outer sheet; re: rectum; ve: ventricle. Scale bars: 50 μm a-f; 200 μm g; 20 μm inset in g.

3.4.2. Ultrastructure

3.4.2.1. Embryogenesis of A. pegreffii

The embryogenesis described herein is based on eggs collected on day 1 and day 6 of incubation. However, due to asynchronous development and hatching, the two time points could have contained eggs at different stages of development.

The eggshell consists of the outermost vitelline layer, a thick central chitin layer, and an innermost lipid layer. The embryo is embedded in the thick electron-light substance enveloped by the plasma membrane, in which discharge of double-membrane vesicles (400 nm in radius) is sporadically observed (**Figure 26A**). The unfertilized egg is acellular with numerous vacuoles with varying contents: electron dense, -light, granular, receding or absent (**Figure 26B**). In an early blastula, a blastocoele is surrounded by six blastomeres that open into the lumen. On the opposite side, a cluster of five cells with a discrete rim of cytoplasm and a few small, electron-dense mitochondria is framed by two large cells (**Figure 26C**). While one cell has a cytoplasm with elongated electron-dense mitochondria, vesicles and multivesicular bodies, the other has a network of convoluted tubular cisternae and sparse perilemmal vesicles (**Figure 26D**). The early gastrulation stage shows somatic cells of the putative ectoderm with a large oval nucleus at the periphery, numerous ribosomes, relatively small mitochondria, and accumulated glycogen (**Figure 26E**). Large empty vacuoles or vacuoles with different electron densities, sometimes fusing together, were common in these cells. The presumptive germline cell is located in the middle of the gastrula and has a large and irregular nucleus (**Figure 26F**). In the later gastrula, the number and size of cells increases, as does the abundance of cytoplasmic granules and vesicles, and the number of larger mitochondria (**Figure 26G**). A single conspicuous cell with a network of developing intracytoplasmic ducts connecting to form a lumen is noted, surrounded by cells connected by numerous tight junctions (**Figure 26H**).

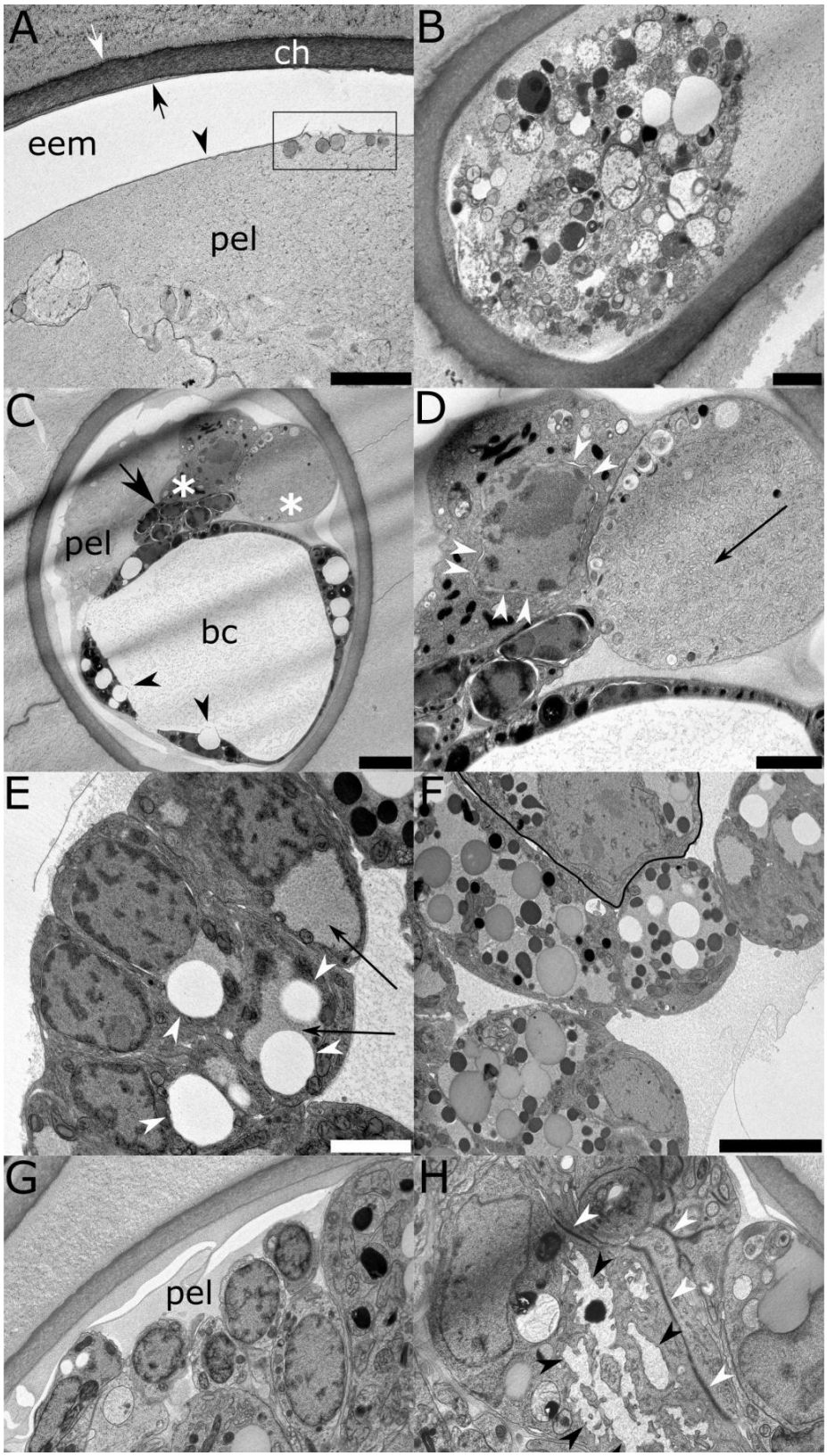

Figure 26. Representative transmission electron micrographs of *Anisakis pegreffii* embryogenesis within the egg: A) Trilaminar eggshell consisting of the vitelline layer (white arrow), chitinous layer (ch), and lipid layer (black

arrow). Below the eggshell, fluid-filled extra-embryonic matrix (eem) envelopes the amorphous peri-embryonic layer (pel) that envelops the embryo (not shown). Peri-embryonic layer is bordered by a thin double-layered permeability barrier (black arrowhead). Black rectangular demarcates the site of double-membrane vesicles secreted into the extra-embryonic layer; B) Acellular unfertilized eggs with numerous granules and vesicles; C) Early blastula with blastomeres surrounding the blastocoele (bc), a cluster of small cells (black arrow), and two larger cells (white asterisk) enveloped in peri-embryonic layer (pel). Note blastomeres' vesicles discharging in the blastocoele (black arrowhead); D) Higher magnification of two larger cells in the early blastula. Note the unregular shape of the large nucleus with distinct nuclear membranes in the left cell (white arrowheads) and the abundant tubular network reminiscent of cisternae in the right cell (black arrow); E) Putative ectodermal cells in the gastrula with an apical rounded nucleus fluid-filled vesicles (white arrowhead), and glycogen accumulations (black arrow) interspersed within the cytoplasm; F) Putative germline cell with a large and irregular nucleus (demarcated by a black line); G) Somatic cells in the late gastrula rich in granules and vesicles, enveloped by periembryonic layer (pel); and H) Putative excretory gland cell, whose cytoplasm is interspersed by branching channels (black arrowhead), reminiscent of the lumen of excretory channel. Note strong tight junctions between somatic cells (white arrowhead). Scale bars: 2 μm a, b, d, e, g, and h; 5 μm c and f.

3.4.2.2. Early ontogenesis of A. pegreffii

The ontogeny described here is based on L2 between 0 and 48 h of age after hatching. In most cases, L2 were observed with the inner cuticle 3 tightly attached to the epidermis and with a loose, enveloping cuticle 2 in the process of shedding.

At the level of the posterior end of the buccal cavity, the excretory channel attaches to the epidermal cell and runs between two anterior nerve cords (**Figure 27A**). The pharynx consists of six firmly connected epithelial cells with a large spherical nucleus rich in ribosomes, vesicles, and few mitochondria. The lumen is closed, Y-shaped, and lined with proteinaceous calix (**Figure 27B-C**). A nerve ring of neurons and neurite bundles consisting of microtubules and neurotransmitter granules surrounds the pharynx (**Figure 27D-F**).

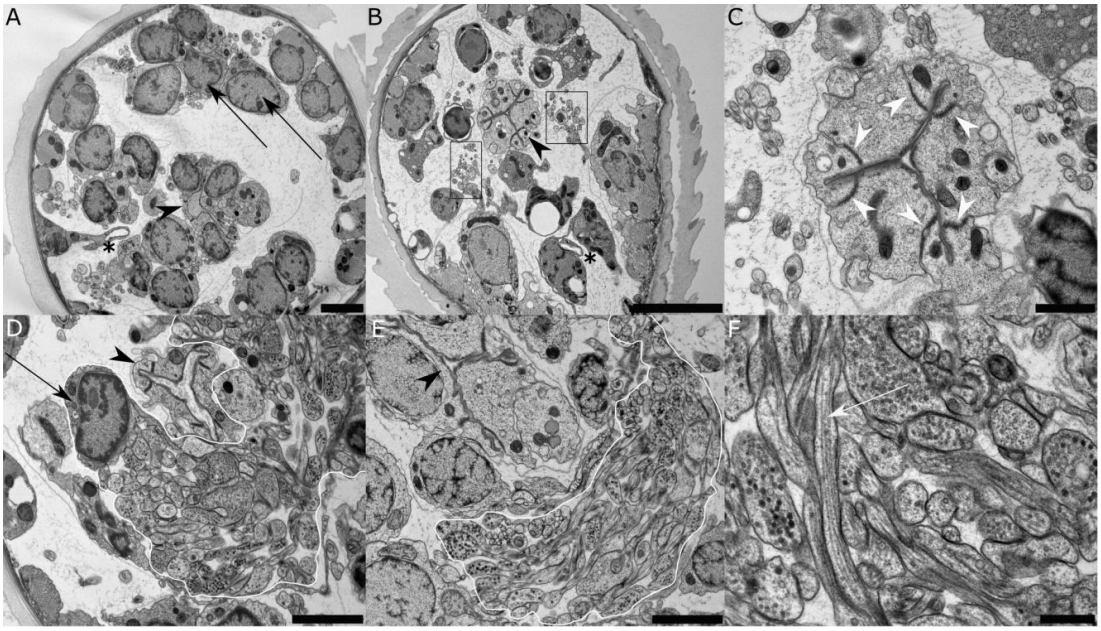

Figure 27. Representative transmission electron micrographs of *Anisakis pegreffii* L2 cross-section at the pharynx level: A) Posterior part of the buccal cavity (black arrowhead) proximal to the excretory channel (black asterisk) and neurons filled with neurotransmitters' granules (black arrows); B) Y-shaped pharynx lumen (black arrowhead) surrounded by two bundles of neurites (black rectangles) and the excretory channel (black asterisk); C) Higher magnification of six epithelial cells forming the pharynx. Note tight junctions between cells (white arrowheads); D) and E) Neurones (black arrow) and neurites (demarcated by a white line) circumventing the pharynx (black arrowhead); and F) Higher magnification of bundles of neurites consisting of neurotubules (white arrow) and granules of neurotransmitters. Scale bars: 500 nm f; 1 μm c; 2 μm a, d, e; 5 μm b.

A putative EGC is already noticeable in L2. The anterior part of its excretory channel passes at the level of the pharynx and shows secretion of what looks like EVs (**Figure 28A-C**). The nucleus of the EGC is larger, irregular in shape, almost lobate, and with a less condensed chromatin than in somatic cells (**Figure 28D-F**). The cytoplasm is rich in ribosomes, few mitochondria, and granules and vesicles of varying size and content. A fluid-filled excretory lumen forms a wide receptacle in the central part of the cell, extending posteriorly above the nucleus (**Figure 28G-I**).

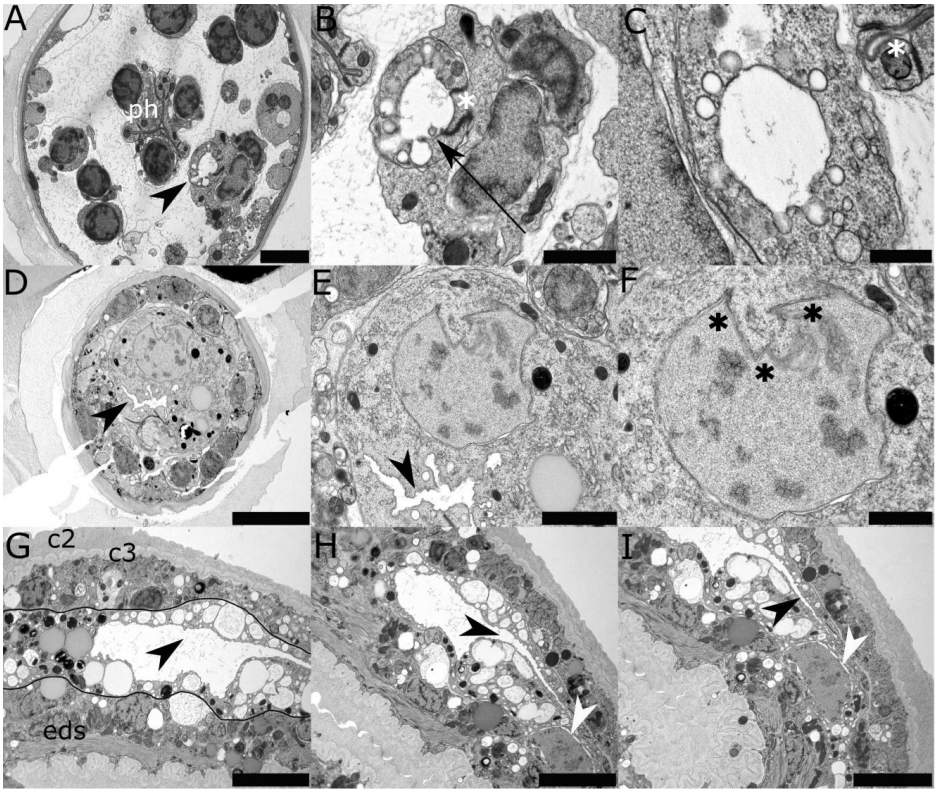

Figure 28. Representative transmission electron micrographs of *Anisakis pegreffii* L2 putative excretory gland cell (EGC): A) Cross section of L2 at the pharynx (ph) level, sided by anterior part of the excretory channel (black arrowhead); B) Higher magnification of the excretory channel filled with fluid and secreted extracellular vesicle (black arrow). Note the apparent junction of the channel to the adjacent somatic cell (white asterisk) that in consequent section in C) disappears, suggesting a transient point of attachment to the epidermis; D) Cross section through body of the putative EGC at the level of excretory channel lumen (black arrowhead); E) Higher magnifications showing irregular lumen of the excretory channel with smaller connecting canaliculi (black arrowhead); F) Large EGC nucleus, with a lobed and irregular appearance (black asterisks); and G)-I) Longitudinal consecutive sections of L2 at the level of EGC (demarcated by a black line), showing anteriorly a receptacle formed by excretory channel (black arrowheads) that posteriorly diminishes in the diameter, directed dorsally to the cell nucleus (white arrowheads). Scale bars: 500 nm c; 1 μm b, f; 2 μm a, e; and 5 μm d, g-i.

Occasionally, various structures are secreted between the cuticle attached to the L2 epidermis (i.e., cuticle 3) and the exsheathed, loose cuticle in the process of shedding (cuticle 2) (**Figure 29A**). These include larger microvesicles (**Figure 29B**), granules with different contents (**Figure 29C-D**), cell-free mitochondria (**Figure 29A, C**) and

EVs (**Figure 29E**). Neurotransmitter release by peripheral neurons to myocytes is also observed in the larval epidermis (**Figure 29F**).

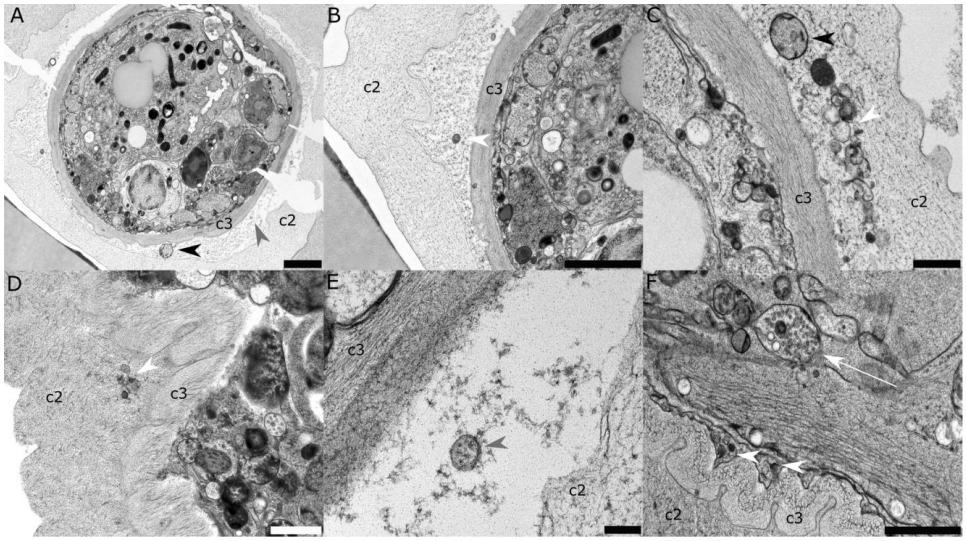

Figure 29. Representative transmission electron micrographs of *Anisakis pegreffii* L2 secretion: A) Cell-free mitochondria (black arrowhead) and extracellular vesicles (grey arrowhead) shed between larval cuticle 3 (c3) and lose cuticle 2 (c2); B) Large microvesicle (white arrowhead); C) and D) Accumulation of granules with different content (white arrowhead) and a cell-free mitochondria within cuticle 2 that is still tightly attached to cuticle 3 at the epidermal surface; E) Extracellular vesicle (grey arrowhead) with double membrane secreted between cuticle 3 and 2; and F) Neuron cell releasing neurotransmitters at the myocytes synapsis in the epidermis (white arrow). Note accumulation of granules (white arrowheads) between epidermis and cuticle 3. Scale bars: 200 nm e; 500 nm c; 1 μm d, f; 2 μm a, b.

Six epithelial cells form the lumen of the putative intestinal tract, which is filled with electron-lucent material. In its part proximal to the pharynx, the intestine is flanked by the posterior part of the EGC, recognisable by the excretory channel, numerous ribosomes, and the rough endoplasmic reticulum (**Figure 30A**). Further aborally, the intestine extends in the lumen and is surrounded by a second layer of densely packed epithelial cells interspersed with neurite bundles and myocytes that form the epidermis (**Figure 30B-C**). At the largest diameter of the gut, the lumen consists of only a single epithelial cell (**Figure 30D**). In the most caudal part, after the anal opening, the body consists of bundles of neurites, a few epithelial cells, and epidermal myocytes (**Figure 30F-H**).

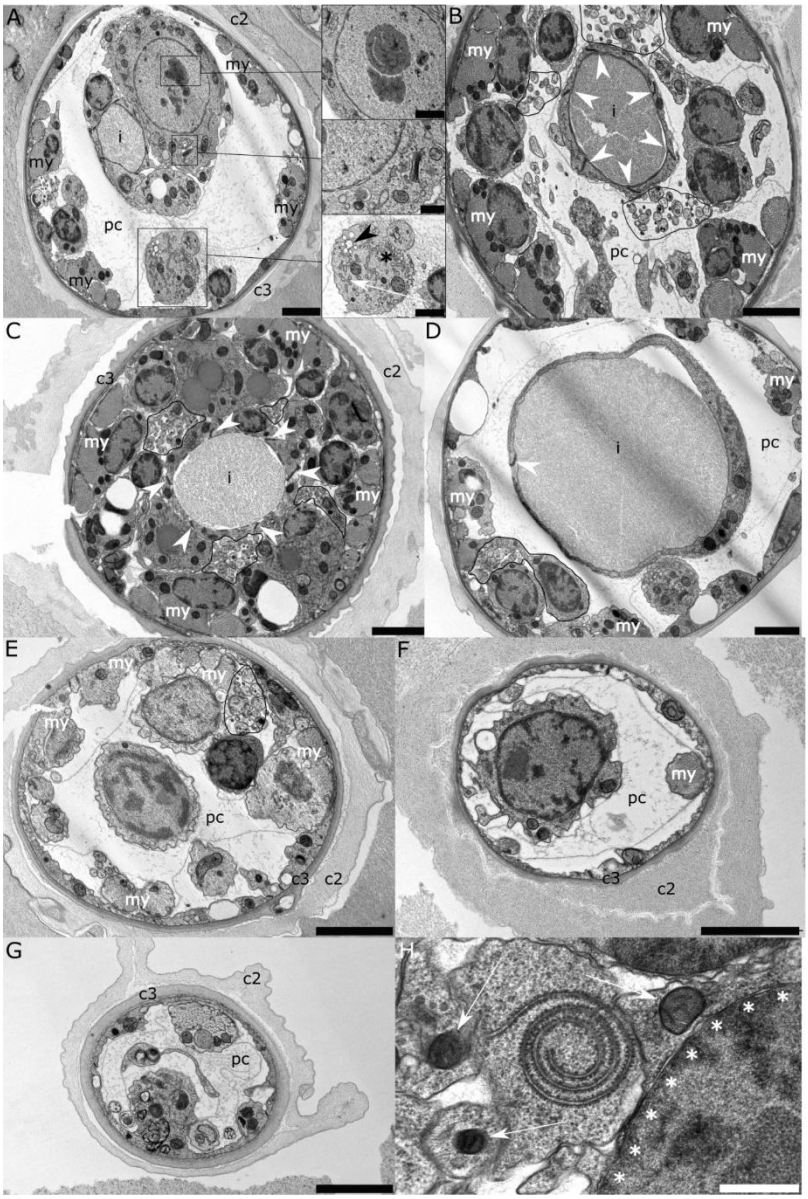

Figure 30. Representative transmission electron micrographs of *Anisakis pegreffii* L2 cross secretions from the intestinal level toward caudal end: A) Putative upper part of the intestine filled with electron-light material. Highlighted in three black rectangles are higher-magnification insets detailing the epithelial cell nucleolus (top inset), Golgi apparatus proximal to nuclear membrane (central inset), and distal part of the excretory gland cell (bottom inset). The latter encompasses the posterior distal part of the excretory channel (black arrowhead), tubular rough endoplasmic reticulum (white arrow), and Golgi apparatus (black asterisk); B) Intestinal lumen made by six epithelial cells connected by tight junctions (white arrowheads), proximal to three bundles of neurites (demarcated by a black line); C) Intestinal lumen surrounded by two layers of epithelial cells. Bundles of neurites (demarcated by a black line) are interspersed between the second layer of epithelial cells. Note the first layer of six epithelial cells that form the lumen (white arrowheads); D) More distal intestinal segment formed by a single epithelial cell (tight junction marked by white arrowhead). Note a neuron and bundles of neurites (demarcated by a black line); E) – G) Caudal segments after the anal opening; and H) A detail of cylindric rough endoplasmic reticulum proximal to the nuclear envelope (white asterisks) and small mitochondria (white arrows). i: intestine; my: myocytes in epidermis; pc: pseudocoelom; c2: larval cuticle 2; c3: larval cuticle 3. Scale bars: 500 nm central inset, h; 1 μm top and bottom inset; 2 μm a-g.

3.5. Karyotype

The protocol applied for preparing chromosome spreads allowed us to obtain *A. pegreffii* chromosomes at different cell stages (**Figure 31A-C**). The mitotic metaphase nuclei (**Figure 31C**) were detected in 3 specimens (two females and one male), although not in large numbers. Thirty mitotic metaphase cells were counted, which had variable numbers of 15 to 20, potentially holocentric, chromosomes. The most frequently recorded number was 18 (43.33%; N=13), suggesting a diploid karyotype formula of 2n=18. The remaining cells showed counts of 20 (23.33%; N=7), 19 (13.33%; N=4), 17 (10% each; N=3), 16 (6.67%; N=2), and 15 (3.33%; N=1) chromosomes.

The positive telomeric signal $(TTAGGC)_n$ in *A. pegreffii* showed consistently variable intensity between chromosomes and between the ends of the same chromosome. A strong interstitial block of telomeric repeats was observed in some chromosomes (**Figure 31D**).

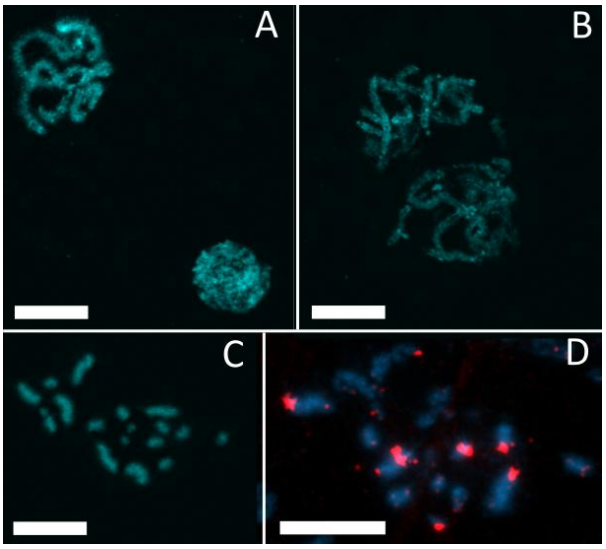

Figure 31. *Anisakis pegreffii* chromosomes at different stages of the cell cycle stained with DAPI (blue) and after fluorescence *in situ* hybridization (FISH) with biotin-labeled telomeric probe (red). A) Cells in interphase (down) and pachytene (up) stages; B) pachytene stage; C) mitotic metaphase. D) Cell in a mitotic metaphase with chromosomes (in blue) showing a positive signal of nematode telomeric sequences (in red). Scale bars = 10 μm.

4. Discussion

4.1. Fecundity

Although expected, this study is the first proof of concept that the culture conditions developed for *A. pegreffii* also support well the development and reproduction of *A. simplex*. Furthermore, it must be taken into account that the *A. simplex* batch we used in the experiment also contained specimens of *A. pegreffii* to a small extent, as the batch originated from a sympatric area of *Anisakis* spp. (i.e., from the North Atlantic).

Several studies have reported on egg production of female anisakids, but they have mainly relied on counting eggs from dissected uteri (McClelland, 1980; Smith, 1988; Marcogliese, 1997; Iglesias et al., 2001). Fecundity data obtained from naturally deposited eggs of nematodes in culture are scarce and only from *A. simplex* (Iglesias et al., 2001; Ugland et al., 2004). Iglesias et al. (2001) reported only two females that laid a maximum of 5,672 and 16,790 eggs per day, which is far from our maximum, 115,694.45 eggs/day/female. However, these authors used a different culture medium based on RPMI-1640 with 20% (v/v) heat-inactivated foetal bovine serum and 1% pepsin, which was inconsistent in achieving oviposition. Ugland et al. (2004) registered oviposition in *A. simplex* females from minke whale cultured in sterile water containing 0.9% NaCl (pH=5-6). These females laid a higher number of eggs than in this study, but 98% of them were laid in the first six days. The saline water cannot sustain nematodes under their physiological conditions, therefore the discharged eggs were probably released from stressed and dying animals in a nutrient-depleted medium.

These are the first data on the fecundity of *A. pegreffii* (i.e., 24,370.96 eggs/day/female average production), which is almost 19% less than the average fecundity of *A. simplex*. However, this interpretation should be taken with caution, as the two experiments lasted for different lengths of time, and we observed increased

production in *A. simplex* batch in the last days (**Figure 21**).

Fertility (or hatching rate) was not assessed in this study, although there are previous reports for both *A. simplex* and *A. pegreffii* at different temperatures, from 3 ºC to 25-27 ºC (88-95% and 1-47% mean hatching rates, respectively; Gomes et al., 2023), as well as for other related nematodes (*C. multipapillatum* in Valles-Vega et al., 2017 and *Hysterothylacium aduncum* in Adroher et al., 2004).

4.2. Culture medium characteristics

Schneider's *Drosophila* medium supplemented with 10% CS, has been shown to be effective in maintaining and developing the early stages of *A. pegreffii* (i.e., from L2 to L3), based on its previous use in the culture of the late developmental stages of *A. pegreffii* (from L3 to adults) (Mladineo et al., 2023). Although the medium was originally intended for the culture of insect cell lines, some studies suggest that it is more suitable for the development of nematodes than media for vertebrate cell lines (Adroher et al., 2004; Mladineo et al., 2023). Given insects and nematodes evolutionary relatedness, particularly their shared experience of ecdysis, a key developmental process (Giribet and Edgecombe, 2017), the medium is expected to support the development of early nematode stages in which multiple ecdyses occur. The low concentration of CS chosen (10%) allowed for balanced L2 growth that correlated with the moulting process, in contrast to the high concentration tested previously (50%), which stimulated rapid growth but was not associated with timely moulting (Mladineo et al., 2023). CS as opposed to foetal bovine serum (most commonly used as a nutrient source) proved to be suitable for both late and early developmental stages of *A. pegreffii* (Mladineo et al., 2023). Moreover, CS has been successfully used for *in vitro* culture of marine trematodes (Fredensborg and Poulin, 2005; Lloyd and Poulin, 2011), suggesting that this serum may be more suitable for the culture of marine helminths than for terrestrial ones.

Some marine invertebrates are osmoconformers that maintain their internal environment isotonic to their external seawater environment. Although there are

few data specifically for marine helminths, it seems that their culture success is influenced by the osmolarity of the medium (De Meester et al., 2011; Xie and Zhang, 2022; Podbielski et al., 2022). Considering that the osmolarity of SDM ranges from 320 to 360 mOsm according to the manufacturer's certificate of analysis, we tested different osmolarities on the early developmental stages of *Anisakis* and found that the best survival rates were obtained with the highest (700 and 800 mOsm) and with SSS (1,200 mOsm) + SDM (1:1). Marine crustaceans, which serve as the first intermediate hosts for anisakids, are also osmoconformers, therefore high osmolarities that mimic biological conditions in krill and other crustaceans allow higher survival rates of early larvae of *A. pegreffii*. Although the use of SSS + SDM is more economical compared to SDM for massive larval rearing, it is limited by the delay in L2 exsheathment compared to the one observed in L2 in high osmolarity SDM, which humpers its application.

4.3. Early development of *A. pegreffii*

To our knowledge, this is the first time that the development of *Anisakis* spp. from L2 to L3 has been achieved *in vitro*. In fact, only for one related taxon, *Contracaecum multipapillatum*, has an attempt been made to develop and characterize *in vitro* hatched larvae (Valles-Vega et al., 2017). However, the experiment was short-termed (25 days) and only ≈2% of the larvae exsheathed. Hatched *C. multipapillatum* and larvae of other anisakids show flicking movements and attachment of detritus before exsheathing, similar to *A. pegreffii* L2 described here (Thomas, 1937; Huizinga, 1966; Davey, 1969; McClelland and Roland, 1974; Højgaard, 1998; Adroher et al., 2004). After exsheathment, *A. pegreffii* shows more serpentine, worm-like movements and adheres less to substrates. We suggest that this is due to better developed somatic myocytes, the properties/composition of the newly obtained cuticle and/or the development of sensory structures that help to avoid detritus. Valles-Vega et al. (2017) observed exsheathment over 25 days only in *C. multipapillatum* larvae maintained at 24 ºC, that started on the 14th day of the experiment. In contrast, *A. pegreffii* started to exsheath on 3rd week of the experiment,

at a lower temperature than *C. multipapillatum* (i.e., 19 ºC). This is consistent with previous reports where higher temperatures were associated with faster development of both nematode eggs and larvae, but with lower larval survival (Brattey and Clarck, 1992; Measures, 1996; Højgaard, 1998; Valles-Vega et al., 2017; Gomes et al., 2023). In addition, the inability to completely shed a piece of cuticle during exsheathment led to larval strangulation in some cases. In *C. multipapillatum*, moulting occurs in the gut of the copepod (Huizinga, 1967), suggesting that host digestive enzymes are involved in this process, as is known for several proteases involved in nematode moulting (reviewed in Page et al., (2014)). *A. pegreffii* moults to L3 in the crustacean haemocoel (Smith, 1983), but since reaching the haemocoel implies previous intestinal or hepatopancreatic migration, digestive enzymes may also be involved in the natural exsheathment process.

In agreement with the observations of Valles-Vega et al. (2017) in *C. multipapillatum*, freshly hatched larvae of *A. pegreffii* already show the boring tooth, pharynx, and developing intestine being ensheathed in the striated cuticle of the L2 stage. The development of the organs begins during postembryonic growth, and after exsheathment all the morphological characteristics of the L3 stage are visible, except for the mucron, which becomes present in older L3 stages. Despite the long duration of the experiment (17 weeks for the last larvae in culture), the L3 did not reach the size of *A. pegreffii* L3 that naturally infects the fish. In this study, the mean length of L3P was 2.27 mm, far below the mean length of L3 from natural fish infections (16.9 mm, Roca-Geronès et al., 2020). Although we detected peristalsis of the intestinal tract, similar to *C. papillatum*, there was no evidence of active feeding, which is expected for L3 surviving in fish in an inactive state of paratenesis (Trumbić et al., 2021).

Finally, it is noteworthy that the older L3 stage differentiates into a large and a small phenotype under the same culture conditions. This is comparable to the appearance of giant *Cenorhabditis elegans* after induction of oocytes fusion by a laser microbeam; the newly formed oocyte continued its normal cleavage pattern after fusion,

suggesting regulation by the uncleaved oocyte (Irle and Schierenberg, 2002). Although we cannot link our observation to the manipulation of *C. elegans* oocytes, it remains to be clarified whether this process is a natural part of *A. pegreffii* ontogeny or whether it is conditioned by an artificial environment.

In summary, *A. pegreffii* moults once in the egg, hatches as L2, subsequently shedding the ensheathed L2 cuticle and becomes L3, with corresponding L3 traits, except for the mucron, which develops in the older L3 stage. Moreover, an additional moult was observed in the older L3 stage, but this was not accompanied by a physiological or morphological shift to the L4 stage. Although an additional moult caused only by nematode growth cannot be ruled out, it is unexpected as the cuticle of nematodes is collagen-based and therefore allows growth during intermediate moulting periods (Lažetić and Fay, 2017). Alternatively, supernumerary moults could occur, as described in *C. elegans*, due to mutations in heterochronic genes (Ambros and Horvitz, 1984; Ambros, 1989).

4.4. Ultrastructure

Milestone studies on the embryonic and postembryonic development of nematodes are based on two model taxa: *Parascaris equorum* (syn *Ascaris megalocephala*) and *C. elegans*. Although they share the same pattern of asymmetric divisions giving rise to somatic founder cells and germline cells, in *P. equorum* a rare phenomenon of chromatin loss due to breakage and degradation of chromatin during early blastomere cleavages results in silencing of somatic genes (Schierenberg and Sommer, 2014). In *C. elegans*, however, each new cell lineage expresses a specific cell cycle rhythm and a fixed cleavage and differentiation programme, including apoptosis, as a distinct cell fate (Schierenberg and Sommer, 2014). In the phylum Nematoda, evolution contributed to the development of substantially divergent cleavage patterns, spatial arrangements, and differentiation of cells, but these features were not followed by corresponding phenotypic changes (Schulze and Schierenberg, 2011). Although taxa share the same characteristic developmental

steps, these are achieved through different cell behavior, so that even within the same species, different scenarios of nematode embryogenesis occur.

Although describing the embryogenesis of *A. pegreffii* is beyond our scope, we have observed snippets of the process that are worth discussing. In the early blastula, for example, the number of blastomeres forming the blastocoele is six, which corresponds to the number of epithelial-like cells forming the pharynx and the intestinal tube of L2 in early postembryonic development. Six blastomeres are supplied with large vacuoles that discharge the liquid content into the newly formed blastocoele cavity. This is consistent with the recognized pattern of blastocoele formation based on successive divisions along the surface plane of the embryo that drive the blastomeres to form a spherical sheet surrounding the cavity (Wolpert et al., 2015). Biophysical approaches in *C. elegans* suggest that the cavity is formed by the growth and coarsening of myriad of micrometric lumens connected by the intercellular space between blastomeres (Verge-Serandour and Turlier, 2021), but we have not been able to observe early-stage embryos to confirm this.

A cell strikingly larger than other somatic cells in *A. pegreffii* L2 was observed 72 h after hatching using confocal microscopy (Mladineo et al., 2023). Based on its size and localization, the authors speculated that the cell will develop into the EGC, but could not provide further evidence due to the lack of specific cell markers. The EGC is one of the most important cells for the survival of parasitic nematodes. It facilitates enzymatic degradation of host tissues, larval penetration and migration, feeding, antigenic interaction with the host immune system, modulation of T-helper and innate immunity axes, and antimicrobial activity within the host gastrointestinal system via excretion and secretion of bioactive compounds released directly in the secretome or packaged in EVs (Baeza et al., 2004; Cotton et al., 2012; Bahlool et al., 2013; McSorley et al., 2013; Fæste et al., 2014; Harnett, 2014; Lee et al., 2017). Here, we described an elongated cell in 48-h-old L2 rich in granules and vesicles, with a large and irregularly shaped nucleus and a channel intersecting the cytoplasm longitudinally and broadening into a central receptacle, reminiscent of

EGC. The anterior L2 cross-sections at the pharyngeal level allowed observation of the excretory channel leaving EGC on its way out through the excretory pore. EVs and fluid secreted in its lumen indicate that already early L2 stage interacts with its environment. The role of the EGC in nematode ecdysis (Davey and Kan, 1968) and the observation that the first moult already occurs in the egg support the assumption that the EGC differentiates during embryonic development and is already functional before larval hatching. Interestingly, we observed an accumulation of EVs, granules, and cell-free mitochondria in the space between the loose L2 cuticle and the tight L3 cuticle on the epidermal surface. While EVs, which were also observed in the excretory channel, could have been expelled through the excretory pore and smaller granules were observed passing through the L3 cuticle, cell-free mitochondria are a rare observation. Indeed, mitochondria are expelled from the cell during developmental processes in some cells, either freely or membrane-enveloped, as both proinflammatory and anti-inflammatory mediators, or in the case of their damage in post-mitotic cells, during a "garbage clearance" (Lyamzaev et al., 2022). Several models have described this process in human and fish cell lines, as well as in squirrel retinal cones, while in *C. elegans* adult neurons release large protrusions containing mitochondria and protein aggregates in the form of exophers during neurotoxic stress (Melentijevic et al., 2017). To our knowledge, this is the first observation of cell-free mitochondria during the ontogeny of a parasitic nematode. Since mitochondria possess properties typical of ancestral bacteria, and their components are recognized as damage-associated molecular patterns capable of activating pattern recognition receptors, their immunomodulatory properties have been demonstrated (Zhang et al., 2010; Krysko et al., 2011; Sarhan et al., 2018). It remains to be proven whether cell-free L2 mitochondria are discharged with the intention of interacting with haemocytes of the first intermediate host.

4.5. Karyotype

Cytogenetic studies have an important role in taxonomy and can help classify

sibling species that are morphologically indistinguishable. This is particularly important in parasitic helminths, where correct identification is essential for accurate epidemiological management and effective treatment (Nielsen et al., 2014; Sofi et al., 2015). Despite the fact that anisakiosis is one of the most important fish-borne parasitic diseases, there are no cytological data for members of the genus *Anisakis*. This could be due to the unavailability of live adults needed for the protocol, which relies mainly on gonadal tissue with its high rate of proliferating cells. To our knowledge, within anisakids, chromosome number data have only been reported for three *Contracaecum* species, namely *C. clavatum*, *C. incurvum*, and *C. spiculigerum* (Goodrich, 1916; Walton, 1924; Walton, 1940). In contrast, the closely related family Ascarididae is well studied (Sofi et al., 2015).

Here we report for the first time a protocol for obtaining *A. pegreffii* chromosome spreads and provide the first chromosome count. Although it is advisable to increase the number of specimens to obtain a higher number of metaphasic cells, which was limiting herein due to the preservation of live adults to study their fecundity, our preliminary result suggests that *A. pegreffii* has a diploid number of 2n=18, possibly holocentric chromosomes (a more detailed technique would be necessary to confirm holocentricity). It is known that most nematodes have holocentric chromosomes (Carlton et al., 2022), and worms with sexual reproduction tend to be diploid (Benazzi, 1982). Our observation of cells with lower or higher chromosome numbers in some cases could be explained as an artefact of spreading, inherent to the procedure. Low numbers may result from the loss of some chromosomes in the metaphases, especially the smaller ones. Conversely, higher chromosome numbers may be the result of chromatids splitting into two parts (Orosová et al., 2021).

We have validated the proof of concept of nematode telomeric probe $(TTAGGC)_n$ in *A. pegreffii*, demonstrating the presence of a conserved repetitive telomeric sequence also in a marine nematode. The telomeric sequence of nematodes was discovered in *Ascaris suum* (Muller et al., 1991) and has only been studied in two subgroups of

nematodes, Rhabditida and Ascaridida (Traut et al., 2007). However, we observed variability in signal intensity in the telomeric endings of *A. pegreffii* chromosomes, which may be due to hypervariability in the length of telomeric repeats (Starling et al., 1990; Rocco et al., 2001). This is not unusual, as in some cases even a complete absence of telomeric signal can be detected, which is due to low copy number of DNA repeats and/or non-clustered organization of these tandem repeats. To better visualize telomeric signals of *A. pegreffii*, tiramide signal amplification FISH could be used as a suitable approach (Cabral-de-Mello and Marec, 2021). Finally, the presence of interstitial telomeric sequences (tandem repeats of telomeric sequence at intrachromosomal positions) in *A. pegreffii* suggests structural rearrangements of chromosomes during nematode evolution (Lin and Yan, 2008).

The karyological information is not only a useful taxonomic tool but, complemented by PacBio long read sequencing technology in combination with conventional short read sequencing, also enables whole-chromosome assemblies of genomes. This is crucial to fill gaps created by repetitive DNA and to achieve greater accuracy in estimating the genome size of helminths. This is important for translational studies focused on the treatment of neglected helminthiases (Carlton et al., 2022; Doyle, 2022).

GENERAL DISCUSSION

Zoonotic parasites from fish have received little attention compared to other well-known parasitosis, despite their high public health significance and emerging status (Chai et al., 2005; Shamsi, 2020). In this thesis, we have contributed to the knowledge on this topic, which has been divided into two parts addressing two different issues.

Information on the occurrence of emerging waterborne unicellular parasites (EWUP) in marine fish remains still scarce, even non-existent for most of the recognized EWUP. Basic epidemiological data, such as prevalence and host distribution, are essential in assessing whether marine fish could be a new source of transmission for any of these parasites. This is why we focused the first study on investigating the presence of three important EWUP, namely *Cryptosporidium* spp., *Blastocystis* sp., and the main zoonotic microsporidian species, in edible marine fish from the Comunidad Valenciana, western Mediterranean, using molecular techniques. While there has been some prior research on zoonotic *Cryptosporidium* and *Blastocystis* in marine fish (refer to **Table 1** in the Introduction), this is the first time that zoonotic microsporidia have been investigated. We found that although zoonotic species (or subtypes) of all these EWUP were able to come into contact with fish and were found in their digestive systems, the very low prevalences detected limit the risk of acquiring these infections through fish consumption. We provided the first comprehensive assessment of these EWUP in both farmed and wild fish from an important Mediterranean region in terms of fish consumption and aquaculture production. However, the possibility of higher prevalences in other regions and/or temporary windows associated with events that could increase the presence of pathogens in marine waters, for example, related to storms (Steele et al., 2018) or inefficient wastewater treatment (Kvesić et al., 2022), cannot be ruled out. Zoonotic isolates were mostly identified in wild fish (synanthropic or from extractive fisheries), suggesting that restricted feeding and movement of farmed-raised fish could limit the likelihood of contact with zoonotic parasites. In any case, this information contributes to highlighting the importance of suitable handling and

manipulation of fish, especially during cleaning and evisceration, since the risk of fishborne infection needs cross-contamination from the digestive tract to the flesh (Chintagari et al., 2017).

When working with targeted PCR techniques, time and economic constraints often lead to the selection of specific pathogens (Scheifler et al., 2019), as was the case in the first study. To overcome these limitations and to gain a comprehensive understanding of the parasite community within a fish/tissue system, we proposed employing a metabarcoding approach. This method is still an emerging research field within parasitology (DeMone et al., 2020). In the second study we presented a pilot research employing a dietary metabarcoding approach to investigate the parasite community, including zoonotic unicellular parasites, present in two Mediterranean Cupleidae fish, the European pilchard and the round sardinella. Whilst this technique based on the 18S rRNA gene was useful as an exploratory method, mapping out the presence of parasites in a host/tissue system –in this case, the digestive tracts of pilchards and sardinellas- it did not provide sufficient taxonomic resolution and underestimated the presence of rare or less abundant genera/species, such as those with zoonotic potential. Despite this, in addition to *Cryptosporidium* and *Blastocystis*, we also identified an additional potentially zoonotic unicellular parasite, *Kudoa thyrsites*. This first approach has relied on an existing protocol due to the need to include a blocking primer (Vestheim and Jarman, 2008). We proposed improvements to tackle these issues based on the design of new blocking primers for other primer sets that show better coverage for parasitic taxa (Kounosu et al., 2019) (or even using alternative methods, such as the recently developed CRISPR-Cas9 digestion (Goldberg and Owens, 2023)) and on the application of the hierarchical metabarcoding (Moszczynska et al., 2009).

As a whole, in this first part of the thesis, we have not only provided relevant information on the epidemiological situation of three important EWUP in marine fish populations of an important region of the western Mediterranean but have also presented pilot research that shows the potential of metabarcoding as a more

optimal technique for studying parasite communities, including the presence of zoonotic parasites, in marine fish. Cross-sectionally, we have also contributed to the knowledge of *Cryptosporidium* species specific to fish (for example, with the description of new hosts for *C. molnari*) and to the parasitic fauna of two Mediterranean cupleids.

On the other hand, there are marine fish true parasites that are fully recognized as fishborne. We have focused on *Anisakis pegreffii* because of its occurrence and importance in the Mediterranean basin (Mattiucci et al., 2018). Several epidemiological studies have been carried out on it (e.g., Cipriani et al., 2015; Bušelić et al., 2018), with current research most directed towards host-parasite interactions, pathogenesis, diagnosis, and treatment (Cavallero et al., 2022a). In the second part of this thesis -third study- we have contributed to the development of *A. pegreffii in vitro* life cycle, which was still incomplete. We achieved high fecundity by employing the recently developed *in vitro* protocol for obtaining adult *A. pegreffii* (Mladineo et al., 2023), and we presented a protocol for the cultivation and maintenance of hatched larvae up to the third-stage larvae (L3) phase. In this way, we are not only providing new information on *A. pegreffii* early developmental traits but also collecting valuable biological material for future research. Indeed, the biological material obtained in this study was used for two different purposes. On the one hand, chromosome spreads were obtained from reproductive adults. Further research is required to confirm the proposed karyotype; this information could in turn be useful to improve genome assembly for this species (Doyle, 2022). On the other hand, ultrastructural characteristics of eggs and recently hatched second-stage larvae (L2) were investigated. It was observed that the excretory gland cell (EGC) is already developed in L2 of up to 48 h. In addition, we observed extracellular vesicles (EVs) and cell-free mitochondria. The EGC is postulated as the major site for the production of excretory/secretory products (ESPs) and EVs in L3 and therefore plays a key role in the pathogenesis and virulence of *Anisakis* (Messina et al., 2016; Cavallero et al., 2018; Mladineo et al., 2023). Based on these findings,

further proteomic experiments are currently underway.

In conclusion, this thesis provides novel information on the epidemiology of some EWUP in edible marine fish from the western Mediterranean. The findings of Chapter 1 demonstrated very low prevalences of three important EWUP in marine fish from the Comunidad Valenciana, indicating a limited risk of transmission through fish consumption. These results are consistent with the findings in Chapter 2 for European pilchards. Finally, the original work in Chapter 3 provides an *in vitro* protocol for the early development of *A. pegreffii*. This tool can be used downstream for other research aimed at studying the pathogenicity, diagnosis, and therapeutics of *A. pegreffii*, the most important fishborne parasite in the Mediterranean basin.

CONCLUSIONS

1. A low prevalence of *Cryptosporidium* spp. has been detected in marine fish. The prevalence has been higher in farmed fish and wild synanthropic fish, possibly due to higher population densities in offshore fattening farms and their surroundings.
2. New hosts for *Cryptosporidium molnari* have been identified, along with two potential new genotypes, highlighting the high diversity of this parasite in fish.
3. The emerging zoonotic species *Cryptosporidium ubiquitum* has been detected for the first time in two fish specimens, specifically in European sea bass.
4. A very low prevalence of *Blastocystis* sp. has been detected in commercial edible marine fish, with only wild synanthropic fish being affected.
5. Three out of the four subtypes of *Blastocystis* sp. identified, namely ST5, ST6, and ST7, are recognized as zoonotic, with subtypes ST5 and ST6 being reported for the first time in fish.
6. A moderate presence of potentially zoonotic microsporidia species has been detected in commercial edible marine fish for the first time, being higher in wild fish from extractive fisheries than in farmed fish.
7. The highest presence of microsporidia corresponded to Enterocytozoonidae, potentially *E. bieneusi*, followed by *E. hellem/intestinalis*. A more in-depth genetic characterization is necessary to ascertain the actual zoonotic risk.
8. The detection of zoonotic emerging species and/or subtypes of *Cryptosporidium* sp., *Blastocystis* sp., and microsporidia in commercial edible marine fish highlights the potential existence of a zoonotic transmission pathway for these parasites through fish consumption, particularly in the case of wild specimens, a consideration previously underestimated. However, the observed low prevalences suggest a limited risk emanating from the studied fish populations. Nevertheless, it is imperative to factor in fish handling and preparation practices when assessing these risks to mitigate potential cross-contamination.
9. Metabarcoding assays based on the 18S rRNA gene are useful in obtaining a general map of marine fish parasitic communities but can mask the presence of

rare but interesting (e.g. zoonotic) species and do not provide sufficient taxonomic deepness for some groups. A hierarchical metabarcoding approach could be more appropriate.

10. Organisms corresponding to four parasitic classes have been detected in European pilchards and round sardinellas by the metabarcoding assay. Pilchards have shown a significantly higher presence of Cestoda and Myxozoa than round sardinellas.
11. Zoonotic genera/species have been detected in the gastrointestinal "parasitome" of European pilchards and round sardinellas, namely *Blastocystis* sp., *Cryptosporidium* sp., and *Kudoa thyrsites*, although in very low prevalence and proportion of reads. In addition, false negatives for *Blastocystis* sp. and *Cryptosporidium* sp. were detected in the metabarcoding assay.
12. It is possible to attain a high fecundity and sufficient period of fertility from *in vitro* cultured *Anisakis simplex* s.l. and *Anisakis pegreffii*, thereby supplying ample biological material for subsequent experiments.
13. An *in vitro* protocol has been developed that enables the obtention of *A. pegreffii* L3 larvae from eggs laid by cultured adults. These larvae can be maintained in culture for up to 17 weeks.
14. Ultrastructural characterization of recently hatched L2 *A. pregreffii* larvae revealed that the excretory gland cell is differentiated up to 48 h post-hatching. Additionally, L2 larvae discharge extracellular vesicles and cell-free mitochondria.
15. The diploid karyotype formula of *A. pegreffii* is presumably 2n = 18.

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ANNEXES

Supplementary table 1. Cultivated fish sampled.

Species	Sampling date	Location	Number of individuals[a]
European sea bass (*Dicentrarchus labrax*)	14/07/2020	Farm 4	15 (12)
	24/07/2020	Farm 2	14
	15/09/2020	Farm 1	14
	21/10/2020	Farm 2	12 (10)
	05/05/2021	Farm 1	10
	12/05/2021	Farm 3	9
	21/07/2021	Farm 3	10
			84 (79)
Gilthead seabream (*Sparus aurata*)	09/09/2020	Farm 4	15 (14)
	15/10/2020	Farm 4	15 (14)
	07/10/2021	Farm 1	9 (8)
			39 (36)
Meagre (*Argyrosomus regius*)	29/09/2020	Farm 2	13 (12)
	07/10/2020	Farm 3	11
			24 (23)
Total cultivated fish			**147 (138)**

[a]Total number of individuals sampled, which coincides with the number of individuals analyzed for *Cryptosporidium* spp. and *Blastocystis* sp. The number of individuals analyzed for Microsporidia is indicated in brackets when different.

Supplementary table 2. Wild synanthropic fish sampled.

Species	Sampling date	Location	Number of individuals
Garfish (*Belone belone*)	15/09/2020	Farm 1	**3**
Bogue (*Boops boops*)	14/07/2020	Farm 4	1
	09/09/2020	Farm 4	1
	15/09/2020	Farm 1	1
	15/10/2020	Farm 4	2
	12/05/2021	Farm 3	1
			6
Thicklip grey mullet (*Chelon labrosus*)	13/07/2021	Farm 3	**1**
European seabass (*Dicentrarchus labrax*)	14/07/2020	Farm 4	2
	24/07/2020	Farm 2	1
	09/09/2020	Farm 4	2
	15/10/2020	Farm 4	5
			10
Sheephead bream (*Diplodus puntazzo*)	13/07/2021	Farm 3	**1**
White seabream (*Diplodus sargus*)	24/07/2020	Farm 2	1
	21/10/2020	Farm 2	3
			4
Common two-banded seabream (*Diplodus vulgaris*)	14/07/2020	Farm 4	2
	24/07/2020	Farm 2	2
	29/09/2020	Farm 2	1
	21/10/2020	Farm 2	2
	07/10/2020	Farm 3	4
	12/05/2021	Farm 3	3
	13/07/2021	Farm 3	2
			16
Red mullet (*Mullus barbatus*)	12/05/2021	Farm 3	**1**
Axillary seabream (*Pagellus acarne*)	24/07/2020	Farm 2	6
	29/09/2020	Farm 2	11
	07/10/2020	Farm 3	7

	13/07/2021	Farm 3	1
			25
Common pandora (*Pagellus erythrinus*)	07/10/2020	Farm 3	1
	15/10/2020	Farm 4	1
	21/10/2020	Farm 2	1
	12/05/2021	Farm 3	1
			4
Round sardinella (*Sardinella aurita*)	09/09/2020	Farm 4	7
	15/09/2020	Farm 1	3
	15/10/2020	Farm 4	2
	12/05/2021	Farm 3	2
	13/07/2021	Farm 3	1
	07/10/2021	Farm 1	10
			25
Chub mackerel (*Scomber japonicus*)	24/07/2020	Farm 2	1
	13/07/2021	Farm 3	2
			3
Comber (*Serranus cabrilla*)	14/07/2020	Farm 4	**1**
Gilthead seabream (*Sparus aurata*)	24/07/2020	Farm 2	1
	29/09/2020	Farm 2	1
			2
Blotched picarel (*Spicara maena*)	14/07/2020	Farm 4	6
	09/09/2020	Farm 4	2
	05/05/2021	Farm 1	3
			11
Black seabream (*Spondyliosoma cantharus*)	14/07/2020	Farm 4	**1**
Pompano (*Trachinotus ovatus*)	15/09/2020	Farm 1	1
	07/10/2020	Farm 3	1
			2
Mediterranean horse mackerel (*Trachurus mediterraneus*)	14/07/2020	Farm 4	1
	24/07/2020	Farm 2	3
	09/09/2020	Farm 4	4
	15/09/2020	Farm 1	2

	29/09/2020	Farm 2	2
	15/10/2020	Farm 4	1
	21/10/2020	Farm 2	6
	05/05/2021	Farm 1	6
	12/05/2021	Farm 3	1
	13/07/2021	Farm 3	5
			31
Total synanthropic fish			**147**

Supplementary table 3. Wild fish sampled from extractive fisheries.

Species	Sampling date	Location	Number of individuals[a]
Argentine (*Argentina sphyraena*)	02/06/2021	Fish market 4	**1**
Meagre (*Argyrosomus regius*)	02/06/2021	Fish market 4	**1**
Imperial scaldfish (*Arnoglossus imperialis*)	02/06/2021	Fish market 4	**1**
Bogue (*Boops boops*)	15/03/2021	Fish market 2	**4 (3) (3)**
Red gurnard (*Chelidonichthys cuculus*)	29/06/2021	Fish market 3	**1**
Spotted flounder (*Citharus linguatula*)	30/03/2021 02/06/2021 29/06/2021	Fish market 3 Fish market 4 Fish market 3	1 (0) (1) 1 3
			5 (4) (5)
European conger (*Conger conger*)	09/03/2021	Fish market 4	**2**
Annular seabream (*Diplodus annularis*)	15/03/2021	Fish market 2	**2**
Common two-banded seabream (*Diplodus vulgaris*)	02/06/2021	Fish market 4	**1**
Blackbelly rosefish (*Helicolenus dactylopterus*)	02/06/2021	Fish market 4	**1**
Brown wrasse (*Labrus merula*)	02/06/2021	Fish market 4	**1**
Large-scaled gurnard (*Lepidotrigla cavillone*)	02/06/2021	Fish market 4	**1**
Blackbellied angler (*Lophius budegassa*)	02/06/2021 29/06/2021	Fish market 4 Fish market 3	1 1
			2
European hake (*Merluccius merluccius*)	09/03/2021 15/03/2021 02/06/2021 29/06/2021	Fish market 4 Fish market 2 Fish market 4 Fish market 3	3 2 1 5
			11
Blue whiting (*Micromessistius poutassou*)	09/03/2021 30/03/2021 02/06/2021	Fish market 4 Fish market 3 Fish market 4	2 2 1

			5
Red mullet (*Mullus barbatus*)	09/03/2021	Fish market 4	1
	15/03/2021	Fish market 2	1
	30/03/2021	Fish market 3	3
	02/06/2021	Fish market 4	1
	29/06/2021	Fish market 3	3
			9
Surmullet (*Mullus surmuletus*)	09/03/2021	Fish market 4	2
	30/03/2021	Fish market 3	1
			3
Axillary seabream (*Pagellus acarne*)	15/03/2021	Fish market 2	**1**
Common pandora (*Pagellus erytrinus*)	15/03/2021	Fish market 2	4 (3) (4)
	30/03/2021	Fish market 3	1
	02/06/2021	Fish market 4	1
			6 (5) (4)
African armoured searobin (*Peristedion cataphractum*)	09/03/2021	Fish market 4	**1**
Greater forkbeard (*Phycis blennoides*)	09/03/2021	Fish market 4	3
	30/03/2021	Fish market 3	4
	02/06/2021	Fish market 4	1
			8
Forkbeard (*Phycis phycis*)	02/06/2021	Fish market 4	**1**
Salema (*Sarpa salpa*)	02/06/2021	Fish market 4	**1**
Atlantic mackerel (*Scomber scombrus*)	09/03/2021	Fish market 4	2
	15/03/2021	Fish market 2	1
	14/06/2021	Fish market 1	19
			22
Lesser spotted dogfish (*Scyliorhinus canicula*)	09/03/2021	Fish market 3	**3**
Comber (*Serranus cabrilla*)	09/03/2021	Fish market 4	**1**
Brown comber (*Serranus hepatus*)	09/03/2021	Fish market 4	1
	30/30/2021	Fish market 3	1
			2
Greater weever (*Trachinus draco*)	30/03/2021	Fish market 3	**1**

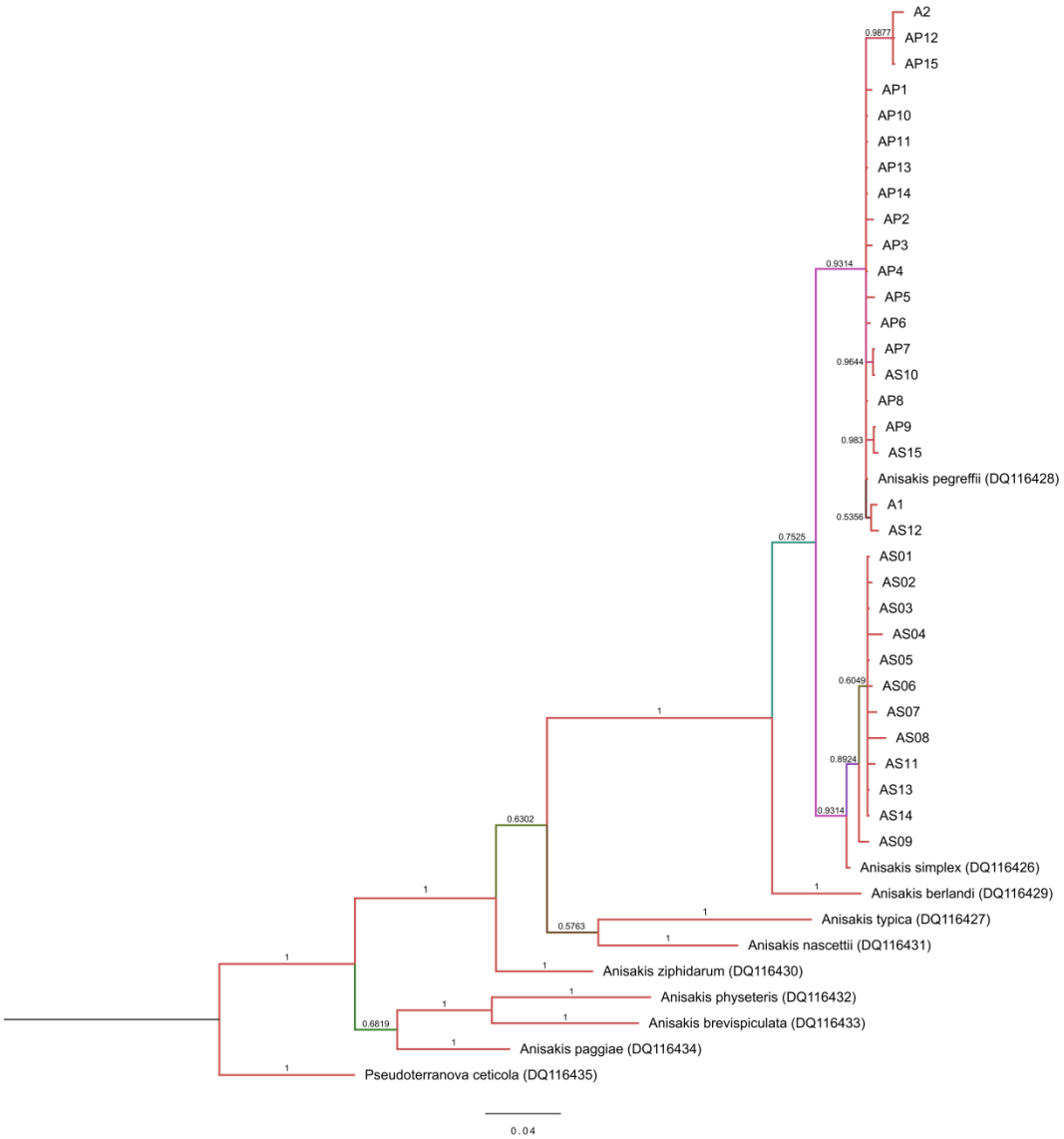
Mediterranean horse mackerel (*Trachurus mediterraneus*)	15/03/2021 02/06/2021	Fish market 2 Fish market 4	2 1
			3
Pouting (*Trisopterus luscus*)	09/03/2021 15/03/2021 30/03/2021 02/06/2021 29/06/2021	Fish market 4 Fish market 2 Fish market 3 Fish market 4 Fish market 3	1 2 6 (5) (6) 1 2
			12 (11) (12)
Stargazer (*Uranoscopus scaber*)	02/06/2021	Fish market 4	**1**
Total fish from fish markets			**114 (110) (113)**

[a]Total number of individuals sampled. The number of individuals analyzed for *Cryptosporidium* spp. and *Blastocystis* sp. is indicated in the first bracket when different. The number of individuals analyzed for Microsporidia is indicated in the second bracket when different.

Supplementary table 4. Parasites from fish dietary organisms identified at the level of genus or species.

Phylum, Class	Genus or Species	Reads	European pilchards (+/total)	Round sardinellas (+/total)	Host prey type
Apicomplexa, Conoidasida	*Lecudina*	46	1/21	0/20	Polychaets
	Polyplicarium curvarae	186	0/21	4/20	Polychaets
	Rhytidocystis	152	1/21	0/20	Polychaets
	Selenidium	268	3/21	1/20	Polychaets
	Selenidium fallax	75	0/21	1/20	Polychaets
Arthropoda, Maxillopoda	*Microsetella norvegica*	2,287	1/21	1/20	Ctenophores
	Sapphirina	433	1/21	2/20	Thaliaceans
Cercozoa, Ascetosporea	*Paradinium*	410	5/21	4/20	Copepods
Cercozoa, Imbricatea	*Pseudopirsonia*	48	0/21	2/20	Diatoms
Ciliophora, Intramacronucleata	*Mesanophrys carcini*	42	0/21	1/20	Crabs
Dinoflagellata, Dinophyceae	*Blastodinium*	27,183	14/21	13/20	Copepods
	Blastodinium contortum	3,995	7/21	5/20	Copepods
	Blastodinium galatheanum	40	1/21	0/20	Copepods
	Blastodinium mangini	3,296	0/21	3/20	Copepods
	Chytriodinium	484	4/21	0/20	Copepods
Dinoflagellata, Syndiniophyceae	*Amoebophrya*	3,172	8/21	9/20	Dinoflagellates
	Duboscquella	210,284	21/21	17/20	Ciliates
	Euduboscquella crenulata	922	3/21	1/20	Ciliates
	Syndinium	45	3/21	0/20	Copepods and radiolarians
	Thalassomyces	48	1/21	0/20	Crustaceans
Holozoa, Ichthyosporea	*Abeoforma whisleri*	52	1/21	2/20	Mussels (*Mytilus* sp.)
	Pirum gemmata	410	6/21	9/20	Peanut worms (*Phascolosoma agassizii*)

Stramenopiles, Bigyra	*Aplanochytrium*	360	0/21	6/20	Sea fan (*Gorgonia ventalina*)
	Solenicola setigera	336	5/21	2/20	Diatom (*Leptocylindrus mediterraneus*)
Stramenopiles, Peronosporomycetes	*Phytophthora*	58	0/21	2/20	Eelgrass (*Zostera marina*)



Supplementary figure 1. Rooted phylogenetic tree inferred by Bayesian analysis of mitochondrial cytochrome c oxidase 2 (*cox2*) fragments of *Anisakis* spp. from *in vitro* cultures, with posterior probabilities given for each node.
Notes: to construct the tree, the HKY+I+G model with a gamma-shape parameter (0.68) and a proportion of invariable sites (0.575) was used, as TrN+G is not available in MrBayes as the best substitution model selected by jModeltest 2.1.7 (Darriba et al., 2012[1]). Four incrementally

[1] Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9, 772-772. https://doi.org/10.1038/nmeth.2109

heated Markov chains were run for 2,000,000 generations, sampling every thousandth generation, and discarding 25% of the samples as relative burnin. A consensus tree with 50% majority rule was constructed and visualized in FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). *Pseudoterranova ceticola* (DQ116435.1) was used as an outgroup to root the tree. The tree corresponds to the well-established topology of the genus, except for the grouping of *A. nascettii* and *A. typica*, albeit with low probability. The branch comprising *A. pegreffii* has a shallow topology with no defined groups. Most specimens are located on the main branch, except for a small, separate cluster comprising three specimens. In contrast, the *A. simplex* branch shows some degree of substructuring, with only the reference sequence of *A. simplex* (DQ116426) placed on the main branch.