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Cryptic Reservoirs of Micro-Eukaryotic Parasites in Ecologically Relevant Intertidal Invertebrates from Temperate Coastal Ecosystems Unveiled by a Combined Histopathological, Ultrastructural, and Molecular Approach

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I. INTRODUCTION

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Most existing eukaryotes including extant ones are singled celled organisms, many of which belong to lineages that diverged early in the eukaryotic domain (O´Malley et al. 2013, Keeling & Burki 2019). Gathered under the popular taxon **Protista** Haeckel 1866, these microscopic organisms hardly share in common anything else than a unicellular level of organization and a membrane-bound nucleus (Margulis et al. 2000, Adl et al. 2005). In fact, neither this term nor its earlier counterpart, **Protoctista** Hogg 1860, are longer recognized by cladistic classifications, including the one agreed by the International Society of Protistologists - ISOP (Adl et al. 2019). The reason is that both clades are paraphyletic, as they exclude the other eukaryote groups (animals, plants, fungi) with which they share a common ancestor (LECA) the "*Last Eukaryotic Common Ancestor*" (O'Malley et al. 2019). However, and in line with other polyphyletic taxa such as **algae**, **amoebae**, or **protozoa**, the term remains useful to refer, though informally, to those eukaryotic organisms that don´t fall within the traditional Kingdoms Animalia, Plantae, and Fungi (Caron et al. 2009). These and other widespread non-monophyletic terms will be used throughout the text for convenience, but spelled without capitalization, following guidance by the ISOP (Adl et al. 2005).

The concern for classifying organisms into groups based on a shared common ancestor (cladistic systems) is particularly apparent in protistology, which having to deal with minute and often uncultured organisms, seeks out as much ecological, ultrastructural, and cell-biological information as possible from close relatives (Taylor 1999, Keeling et al. 2005). In this context, phylogenetic trees represent very useful tools to depict evolutionary relationships between organisms along a range of time scales (Keeling & Burki 2019). The first phylogenetic trees aimed to reconstruct the evolutionary history of living organisms based on morphological and trophic characteristics (Baldauf 2008). However given their small size, wealth of convergent characters, and mixed trophism the task rapidly proved more intricate for microscopic eukaryotes than for their macroscopic cousins (Leander 2020). Therefore, the classification of protist species and lineages has varied a lot in the last 150 years, leaving behind a profuse trail of evolutionarily unsatisfactory assemblages and terms (Cavalier-Smith 1981). In here, we review the main classifications and terminology used to classify protists with respect to the rest of eukaryotes; with the objective of establishing connections between better known groups and currently accepted monophyletic clades, which will be used throughout the text.

1.1. Classification and phylogeny of protists through history

Unlike their much larger multi-cellular counterparts (animals, plants, and fungi), the mere existence of protists was unsuspected until the XVII century, when primal microscopes allowed to observe microorganisms for the first time (Fig. 1A). Until then, all living things were classified as animals or plants (although minerals were often included too), two major groups of organisms with very distinct modes of nutrition and motion. On the one hand, the photosynthetic rooted higher plants, on the other hand, the food-ingesting motile animals (Whittaker 1969). So apparent and immovable was this dichotomy that even macroscopic Fungi were classified within plants based on their apparent sessility and development of root-like structures (Dube 2013). Within this framework, the first "animalcules" (lat. "Animalculum" = tiny animals) observed to the microscope by Antonie van Leeuwenhoeck in 1670-1680 (reviewed by Lane. 2015), were largely obviated by Carolus Linnaeus in his pioneer classification of life (Fig. 1B). From the >9000 species described and classified by Linnaeus in his Systema Naturae (Linnaeus 1758, 1759), only a handful (*Volvox* sp., *Chaos* sp., *Vorticella* sp.) were unicellular; being classified within "Vermes" in the just erected Kingdom Animalia.

For over a century, the mounting diversity of microscopic organisms discovered (including minute animals, algae, fungi, and protists) was subdivided, based on similarities to the two existing Kingdoms, as protozoa (animal-like) Goldfuss 1818 or protophyta (plant-like) Endlicher 1830. However, the number of microorganisms alternating between flagellated, ameboid, and non-motile stages that combined photosynthetic, ingestive, and absorptive nutrition modes commenced to pile up, rendering differentiation increasingly intricate (Whittaker 1969). Influenced by the cell theory (Schleiden 1838, Schwann 1847), in 1860 John Hogg proposed the foundation of a new Kingdom, protoctista (Fig. 1C), to include all unicellular organisms of both plants and animals (Hogg 1860). While his classification suggested a "primigenal" status (Gr. *Protos*- "very first", *ktista* "established beings"), it was not evolutionary (review by Scamardella 1999). Actually, it was Ernst Haeckel (1866) the first one to crystallize Darwin´s evolutionary ideas (1859) into a phylogenetic tree (Fig. 1D). In it, unicellular forms shared a common ancestor with representatives of the animal and plant Kingdoms. Despite the paraphyly of Haeckel´s Kingdom protista, as it included bacteria (moneres) and microscopic metazoans (sponges, myxozoans), Haeckel established the foundations for the evolutionary study of protistan diversity (Olsson et al. 2017).

By the end of the 19th century, the belief that protista was too polyphyletic to be considered a single Kingdom was rather extended, especially when bacteria were included (Rothschild 1989)

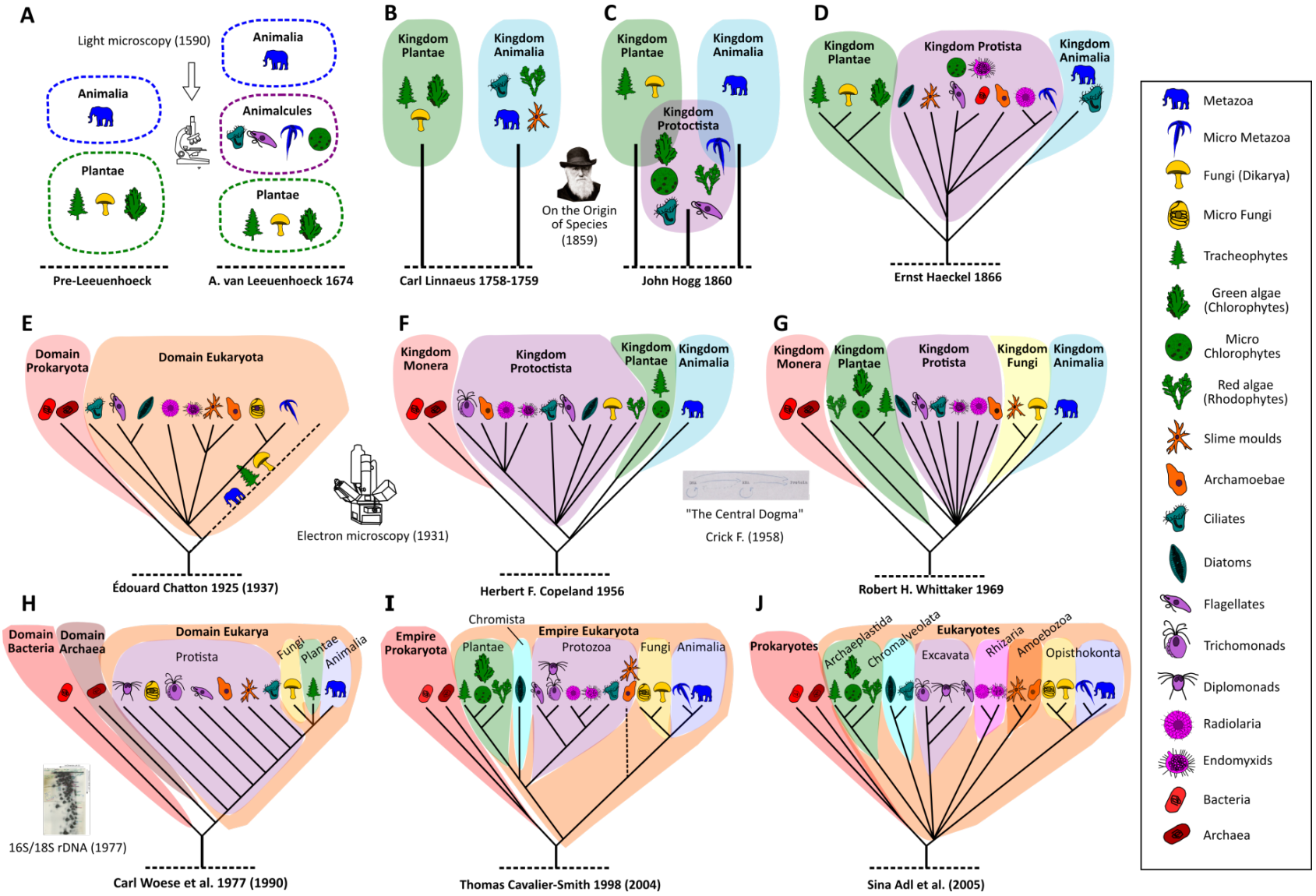
A
Light microscopy (1590)
Animalia
Plantae
Animalcules
Plantae
Animalia
Pre-Leeuenhoeck
A. van Leeuenhoeck 1674
B
Kingdom Plantae
Kingdom Animalia
On the Origin of Species (1859)
Carl Linnaeus 1758-1759
C
Kingdom Plantae
Kingdom Animalia
Kingdom Protoctista
John Hogg 1860
D
Kingdom Plantae
Kingdom Protista
Kingdom Animalia
Ernst Haeckel 1866
E
Domain Prokaryota
Domain Eukaryota
Electron microscopy (1931)
Édouard Chatton 1925 (1937)
F
Kingdom Monera
Kingdom Protoctista
Kingdom Plantae
Kingdom Animalia
"The Central Dogma"
Crick F. (1958)
Herbert F. Copeland 1956
G
Kingdom Monera
Kingdom Plantae
Kingdom Protista
Kingdom Fungi
Kingdom Animalia
Robert H. Whittaker 1969
H
Domain Bacteria
Domain Archaea
Domain Eukarya
Protista
Fungi
Plantae
Animalia
16S/18S rDNA (1977)
Carl Woese et al. 1977 (1990)
I
Empire Prokaryota
Chromista
Plantae
Empire Eukaryota
Protozoa
Fungi
Animalia
Thomas Cavalier-Smith 1998 (2004)
J
Prokaryotes
Archaeplastida
Chromalveolata
Eukaryotes
Excavata
Rhizaria
Amoebozoa
Opisthokonta
Sina Adl et al. (2005)
Metazoa
Micro Metazoa
Fungi (Dikarya)
Micro Fungi
Tracheophytes
Green algae (Chlorophytes)
Micro Chlorophytes
Red algae (Rhodophytes)
Slime moulds
Archamoebae
Ciliates
Diatoms
Flagellates
Trichomonads
Diplomonads
Radiolaria
Endomyxids
Bacteria
Archaea

Figure 1. (Preceding page). Graph summarizing the major classifications of life, with special attention to the position of protists. (**A**) Antoine van Leeuwenhoeck (1674) is credited to be the first one to have described protists. The division between animals and plants existed, but not as Kingdoms. (**B**) Graphical interpretation of Linnaeus´ classification in *Systema Naturae* (1758, 1759). **(C)** Graphical adaptation from Hogg 1860. **(D)** Adapted from Haeckel 1866. **(E)** Graphical adaptation from Chatton 1925. **(F)** Adapted from Copeland 1956. **(G)** Adapted from Whittaker (1969). **(H)** Modified from Woese et al. 1990. **(I)** Adapted from Cavalier-Smith 2004. **(J)** Graphical interpretation from Adl et al. 2005. Legend**:** Drawings of protists in the trees and the legend correspond to actual taxa/species described by the authors, not necessarily the lineage specified at the time. The colours used for the protist depictions correspond to monophyletic clades established by Adl et al. 2005. For instance, ciliate species were actually described by Leeuwenhoeck and already classified by Linnaeus and Haeckel, despite clade Ciliophora not being named as such at the time.

From the alternative classifications that arose to Haeckel´s three-Kingdom system, Otto Bütschli´s (1880-1889) was the most significant one (Corliss 1998, Caron et al. 2012). The classification, polyphyletic itself, divided protists into **sarcodina** (ameboid organisms), **sporozoa** (parasitic organisms), **mastigophora** (flagellated organism), and **infusoria** (ciliated organisms), essentially recovering the concept of protozoa, which was elevated to Kingdom level (Bütschli 1887). The clade excluded single-celled photosynthetic organisms (increasingly regarded to form a continuous with multicellular chlorophytes) and bacteria, observed to have a distinct cellular organization and replication (Whittaker & Margulis 1978). Despite its polyphyly, Bütschli´s classification was solidly established through the first half of the XX century, and even nowadays remains popular among non-protistologist (Adl et al. 2005). His work also influenced Édouard Chatton´s classification of life (1925), which distinguished the characteristic cellular organization of bacteria in opposition to the rest of eukaryotes (Fig. 1E); establishing a rank above Kingdom, the Domain (Stanier 1961).

There was a major leap in protistology during the second half of the 20th century, when the extended use of electron microscopy allowed discerning great morphological variation among microscopic eukaryotes (Taylor 2003). Such escalation of phenotypic diversity made possible the assignment of many species to cohesive phyletic groups, many of which persist today (Keeling & Burki 2019). However, the evolutionary relationship between major protist lineages remained ambiguous and with it the monophyly of the Kingdom (Sogin & Silberman 1998), as it can be noticed in Copeland´s classification of 1956 (Fig. 1F). Apart from distinguishing between prokaryotes (monera) and eukaryotes (protoctista, Plantae, and Animalia), his four-Kingdom system disregarded the unicellular/multicellular dichotomy as a phylogenetic criterion (Copeland 1956).

This major step forward was acknowledged by Whittaker (1969), when he suggested the, perhaps, most familiar of all classifications of life (Fig. 1G). Still widely used, the five-Kingdom system considered not only morphological and cell-biological, but also ecological traits

(especially nutrition mode) to assess the evolutionary history of living organisms. The classification, which did well grouping Rhodophytes and Chlorophytes into Kingdom Plantae; and separating Fungi from the other Kingdoms (based on a predominantly absorptive feeding approach), was still polyphyletic, most dramatically among protist clades (Margulis 1971). In fact, few derived and many convergent characters had prevented major restructurings to Bütschli´s traditional classification of protozoa at the highest ranks before the appearance of the five-Kingdom classification (Honigberg et al. 1964). On behalf of the Society of Protozoologists, Levine et al. (1980) suggested dividing protozoans into seven phyla: sarcomastigophora, labyrinthomorpha, Apicomplexa, Microspora, Ascetospora, Myxospora, and Ciliophora. Despite the monophyly of several of these clades, they did not represent equivalent ranks, neither were the evolutionary relationships between them specified (Corliss 1994).

Taxonomic relationships among protists, multicellular eukaryotes, and prokaryotes experienced a complete reshuffle with the advent of molecular techniques. The DNA structure had already been unveiled before Copeland´s four-Kingdom system (Watson & Crick 1953); and the DNA-RNA-protein translation process, the "Central Dogma" (Crick 1958), had been put forward before Whittaker´s notorious classification too. Even the enormous potential of DNA to disentangle early evolutionary events had been suggested based on species-specific variations in the DNA structure (Crick 1958, Zuckerkandl & Pauling 1965). However, it took until 1977 for Carl Woese & George Fox to construct the first molecular-based phylogenetic tree (Woese & Fox 1977, Woese et al. 1990). Using just a handful of short nucleotide sequences from prokaryotic and eukaryotic organisms, they projected a three-Domain tree of life. This pioneer molecular phylogeny outlined a significant but until then unnoticed evolutionary difference between prokaryotes, separating them in Domains Archaea and Bacteria. Besides, it suggested greater genetic variation between certain protist clades than between some of the multicellular Kingdoms within Domain Eukarya (Fig. 1H). Thus, previous morphological, ecological, and ultrastructural major classifications of eukaryotes were reasoned to be paraphyletic and uneven in the assignation of higher ranks (Woese et al. 1990).

The following years, the number of organisms to be sequenced and analysed increased from just a handful to hundreds and quickly to thousands (Hillis 1987, Vossbrinck et al. 1987, Maden et al. 1995). Early phylogenetic trees using a single or few conserved genes had enough resolution power to identify relationships within protist lineages and sometimes between them (Sogin 1991). For instance, the six-Kingdom classification of life by Cavalier-Smith (1998) already depicted several unforeseen associations between clades such as, amoeba and slime moulds, diplomonads and parabasalids, or radiolarians and endomyxids (Fig. 1I). Furthermore,

traditional protistan clades Microsporidia and Myxozoa were removed from protozoa and clustered with fungi and animals respectively (Cavalier-Smith 1998). However, even the most balanced and complete 18S-rRNA-based trees were not able to disentangle early occurring diversions between major clades (Cavalier-Smith & Chao 2003).

With the turn of the century, ever longer stretches of DNA were used to increase the phylogenetic signal, and soon "phylogenomic analyses" were common tool to resolve evolutionary relationships between major and early diverging clades (Wolf et al. 2002, Delsuc et al. 2005, Dunn et al. 2008). Early protein-based trees confirmed that genetic variation between protist lineages was greater than that between some of the multicellular Kingdoms (Steenkamp et al. 2006). A human is evolutionarily closer to a Caesar´s mushroom, than a labyrinthulid is to an amoeba (despite both being classified as slime moulds). This epitomized a long-standing struggle for the traditional classification scheme (Phyla, Class, Order...), which had to reconcile having "Kingdoms" within "Kingdoms". Accordingly, the "Kingdom-based" classification of eukaryotic life started by Linnaeus, and revised by several authors (Copeland 1956, Whittaker 1969, Woese and Fox 1977, Cavalier-Smith 1998), was replaced by one formed by "Supergroups" (Simpson & Roger 2004, Adl et al. 2005, Keeling et al. 2005). This unranked classification (Fig. 1J) which intended to be more flexible and easier to modify without a cascade of changes, has been widely adopted by protistologist and continuously being updated (Adl et al. 2012, Adl et al. 2019, Keeling & Burki 2019).

1.2. Current phylogeny of protists within Domain Eukaryota

In the current classification of eukaryotes (Fig. 2), long-existing kingdoms Fungi and Animalia have coalesced to form supergroup Opisthokonta Cavalier-Smith 1987. The clade also includes several unicellular lineages more closely related to animals (Myxozoa, Choanoflagellatea, Corallochytrea, Ichthyosporea) and fungi (Microsporidia, Cryptomycetes, Chytridiomycota, Nuclearia) than to other protist lineages (Cavalier-Smith & Chao 1995, Steenkamp et al. 2006, Adl et al. 2012). Opisthokonts are in turn sister to breviates and apusomonads, two small lineages of flagellated heterotrophs (Karpov & Milnikov 1989, Cavalier-Smith 2004), forming a robust clade named Obazoa Brown et al. 2013. Obazoans share a common ancestor with most protists presenting an amoebic locomotion (amoebas), which are grouped within Amoebozoa Cavalier-Smith 1998.

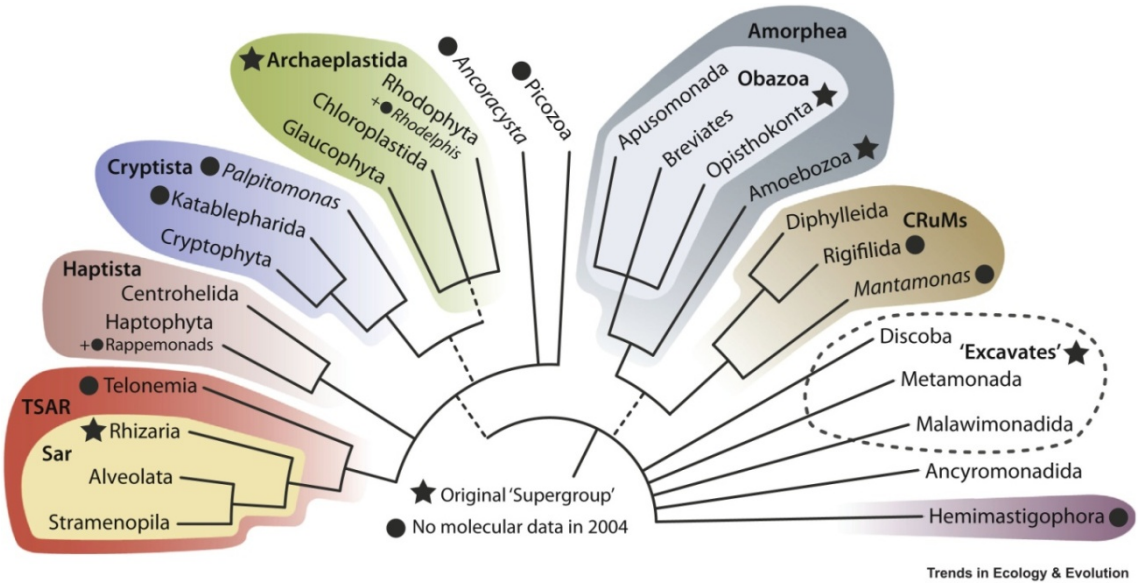

Figure 2. Recent phylogenetic tree of eukaryotes. (from Burki et al. 2020). The consensus tree summarizes the latest phylogenomic studies. Coloured groupings correspond to current "Supergroups". Unresolved branching order among lineages is shown as multifurcations. Broken lines reflect lesser uncertainties about the monophyly of certain groups. Star-shaped forms denote taxa that were considered as supergroups in early versions of the supergroup model (Adl et al. 2005). The circles show major lineages that had no molecular data when the supergroup model emerged.

Green plants (tracheophytes) and algae (charophytes, chlorophytes, trebouxiophytes) group together with red algae (rhodophytes), and glaucophytes forming supergroup Archaeplastida Adl et al. 2005. In contrast, the so called brown algae (phaeophytes) form, together with diatoms, part of the TSAR Strassert et al. 2019 Supergroup, which hosts most of the existing protist diversity (Burki et al. 2007, Bjorbækmo et al. 2020).

Many protist taxa within Supergroups Haptista Cavalier-Smith 2003 and Cryptista Adl et al. 2019 are constituted by photosynthetic algae and ciliated heterotrophs (Cavalier Smith et al. 2015, Adl et al. 2019). Formerly classified as chromalveolates, cryptophytes are likely sister to archaeplastids and central in the study of plastid origin and spread among eukaryotes (Yabuki et al. 2014). Haptophytes, which include among others the well-known coccolithoporids, show in turn, certain attraction for the TSAR supergroup. This majoritarily photosynthetic protists play crucial roles in marine ecosystems and global biogeochemical cycles (Burki et al. 2020). Finally, the evolutionary position and monophyly of excavates, one of the founding Supergroups, is uncertain (Simpson 2003, Burki et al. 2020). The clade, if confirmed, would group very significant and diverse protists lineages such as metamonads and discobids, both parasite-rich lineages (Gull 2001, Cepicka et al. 2006, Kolisko et al. 2010).

2. Evolution of parasitism among protists

2.1. Concepts and evolution of parasitism

A great variety of definitions and usages of the terms parasitism and symbiosis occur in the literature (Martin & Schwab 2013), changing significantly through time and research field (Rohde 2001). In consequence, most of the works dealing to some conceptual depth with parasites/symbionts commence by establishing which of the many views will be applied (Barber & Dingemanse 2010, Esch & Fernandez 2013). One of the most comprehensive definitions of **parasitism** describes it as an "antagonistic symbiosis", where **symbiosis** is understood as: "an intimate association or close union of two dissimilar organisms" (review by Kreier 2013). This conception of symbiosis is a long way from the more traditional "mutually beneficial" association (Lewin 1982), which is now defined as **mutualism** (Bronstein 2015). Drawing up clear-cut boundaries between antagonistic and mutually beneficial symbioses is difficult in the theory, and even more in the practice, as they represent a continuum (Ewald 1987). Besides, it is common among symbiotic organisms to alternate between parasitic and mutualistic behaviours, often while associated to the same individual host (Leung & Poulin 2008). However, a good understanding and consensual usage of this terminology is of vital importance to characterize correctly the myriad of associations existing between organisms of different species (Goff 1982).

Correspondingly, a wide-ranging definition of parasitism defines it as a type of symbiosis, in which one of the two associated animals lives and feeds, temporally or permanently, either in or on the body of the other (Kreier 2013). Some way or another, the concept of "feeding" must be included, because using the host just for transportation is called **phoresy** (White et al. 2017). Different levels of stringency in the interpretation of this word (feed), will result in the scission of parasitism onto **tissue parasitism** and **commensalism**, depending on whether the symbiont feeds directly or not on host tissues (Morris 1992). While the antagonistic effect of commensals on the host is considered to be marginal by definition (Casadevall & Pirofski 2000), they often exert a significant impact to the fitness of the host (Miller et al. 2006), blurring the line with true parasitism. Transience is an important part of the definition too, because although some organisms are exclusively parasitic, the majority of species have free living stages as well (Weinstein & Kuris 2016).

Prefixes "Endo"- and "Ecto"- are used to refer to symbioses (parasitic, commensalistic, mutualistic), in which the smaller organism lives inside or outside the host respectively (e.g. endoparasite, ectosymbiont). Even though ectoparasites might intuitively appear less damaging

to the host than endoparasites, this is not necessarily the case. Significant fitness reduction and major mortality events have been associated to parasites living “on” rather than “in” the host (Rothermel et al. 2008, Csata et al. 2014). For instance, apicomplexan clade Gregarinasina, which is formed exclusively by endoparasites (Rueckert & Devetak 2017), includes a number of species believed to have little or no impact for the host (Bollatti & Ceballos 2014). In contrast, certain ectoparasitic ciliates (TSAR: Alveolata) that grow attached to fish skin and gills, have been shown to be lethal to the host under certain conditions (Khan 2004). Depending on the harm exerted to the host by the parasite, the later can be considered or not a **pathogen** (Méthot & Alizon 2014). At last, those symbionts (including parasites) unable to live apart from their host are thought off as **obligate symbionts**, and those that can survive without the host, **facultative symbionts** (Fisher et al. 2017). It is important to remark that some forms of behavioural parasitism, such as brood parasitism or Kleptoparasitism do not always fit in several of the concepts and terms above described.

Parasitism is a very competitive lifestyle, not only is the host exploited as energy source, but also becomes a very stable habitat for the parasite to inhabit (Combes 2001, Mestre et al. 2020). Even for parasites in the commensalistic side of the spectrum, the host provides a concentrated and often partially processed source of nutrients and energy in comparison to the surrounding environment (Poulin 2011). Thus, selection has favoured those organisms capable of exploiting the resource-rich niche that the host represents (Rhode 1994). A hint of the success of parasitism can be measured as the number of existing parasite species and the number of times that this strategy has evolved (Poulin & Randhawa 2013). According to some of the most sophisticated estimates of global diversity, parasites would account for anywhere from one third to over half of the species on earth (Windsor 1998, de Meeûs & Renaud 2002, Dobson et al. 2008, Poulin 2014), representing the most common consumer strategy among living organisms (Lafferty et al. 2008), including micro-eukaryotes (Fig. 3). For instance, just among apicomplexans, highly specialized animal-infecting parasites (Molnár 2006), at least one species is thought to exist for every single animal species (Morrison 2009). Moreover, parasitism is known to have evolved independently several times in every single eukaryotic supergroup (Poulin & Randhawa 2013, Lukeš et al. 2014). Actually, some groups like protostome animals or red algae have seen over 100 independent jumps from free-living to parasitic lifestyles each (Blouin & Lane 2012, Zrzavý 2013). In contrast, barely any transition to parasitism has been documented among deuterostomes (Chordata and Echinodermata), brown algae, diatoms, euglenids, or haptophytes (Bavestrello et al. 2000, de Meeûs & Renaud 2002, de Vargas et al. 2007, Weinstein & Kuris 2016).

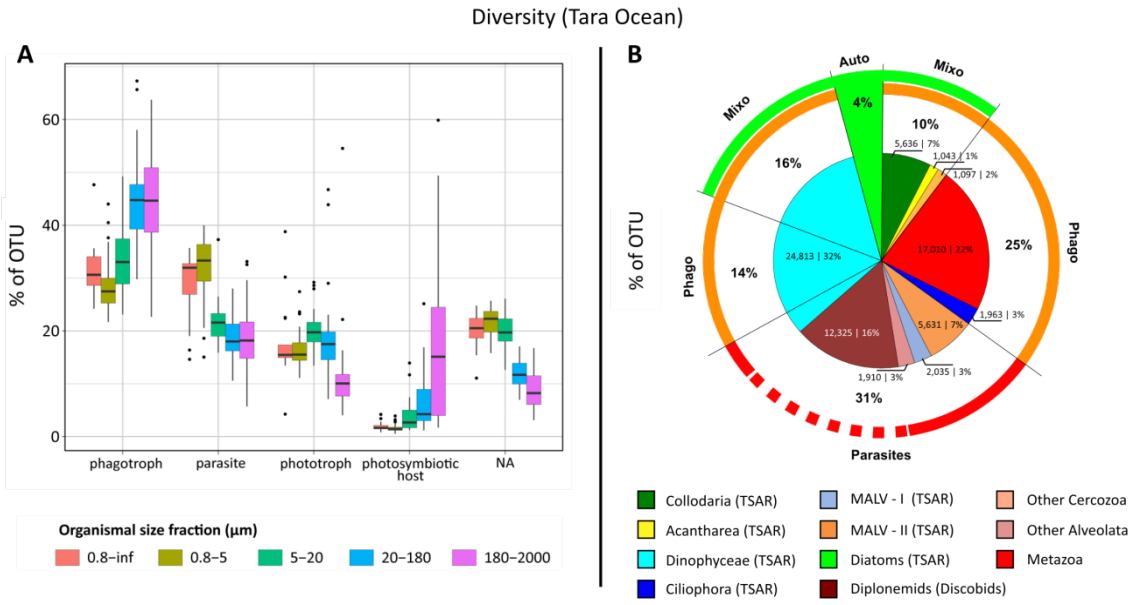

Figure 3: Parasite diversity represented among size fractions and eukaryote clades based on the Tara Oceans V9 rDNA metabarcoding dataset. **(A)** rDNA-based diversity of main trophic modes across organismal size-fractions in photic-zone eukaryotic plankton. Notice the relative diversity (measured as percentage of Operational Taxonomic Units – OTUs) of parasitic eukaryotes in the 0.8 - 5 µM size fraction. **(B)** Pie-charts displaying the contribution of the most diverse planktonic eukaryotic lineages to broad ecological functions: parasitism ("Parasites"), phagotrophy ("Phago"), phototrophy ("Auto"), mixotrophy ("Mixo"), in terms of species richness (number of OTUs) (from de Vargas et al. 2015).

Seemingly, some eukaryotic lineages (including multicellular and unicellular organisms) jump more easily from free-living to parasitic lifestyles than others, although the actual reasons remain elusive (Weestgood et al. 2010, Janouskovec & Keeling 2016). Several non-mutually exclusive hypotheses have been proposed to explain the apparent potential of some lineages to carry out this transition more readily (Luong & Mathot 2019), including fungal hyphae association, predator-prey interaction, close cohabitation, and pre-adaptation (Poulin 2007, Naranjo-Ortiz & Gabaldón 2019). In essence, the first three refer to **opportunity**, understood as frequent interaction between two populations of organisms that don´t eat or harm each other. Additionally, possessing phenotypic, genetic, metabolic, or cell-biological **pre-adaptations** for survival, feeding, and reproduction on the host are thought to be crucial for the parasite-to-be (Poulin 2011). Although the origins of parasitism among protists lineages is, like the group itself, polyphyletic (Baker 1994); these transitions to parasitism, whether they have undergone extensive diversification or not, are of paramount importance to understand the distribution of parasites among current eukaryote Supergroups.

2.2. Parasitic protists in a phylogenetic context

Opisthokonta, the largest of the clades constituting Supergroup Amorphea, includes the traditional Kingdoms **Animalia** and **Fungi** along with their unicellular relatives (Adl et al. 2012).

Representing the bulk of existing species within the **animal lineage** (**Holozoa**), bilaterally organized multicellular organisms show an enormous phenotypic, genotypic, and ecological diversity (Finnerty et al. 2004). Predictably, the number of pre-adapted organisms finding the opportunity to jump to a parasitic type of symbiosis is greater in a clade with over a million described species (Huyse et al. 2005, Mora et al. 2011). Therefore, the existence several parasite-rich phyla (Nematoda, Arthropoda, Platyhelmitha, Acantocephala, Mesozoa), with the ability to infect all major eukaryote Supergroups (Fig. 4), often as pathogens, is hardly surprising (de Meeús & Renaud 2002, Weinstein & Kuris 2016). Early evolving lineages of multicellular animals such as cnidarians, ctenophores, or poriferans have also seen independent transitions to parasitism (Hooper 2005, Haddock 2007, Okamura et al. 2015). The cnidarian clade Myxozoa represents by far the most significant of them. This lineage of obligate animal-infecting parasites is formed by no less than 2000 species (Holzer et al. 2018), including important pathogens of fish (Feist & Longshaw 2006). Traditionally classified within the polyphyletic protist clade Sporozoa (Lom & Dyková 2013), their apparent single-celled structure was more recently shown to be a secondary loss of multicellularity (Cavalier-Smith 2017). Among true unicellular holozoan lineages, Ichthyosporea is the only one to include parasitic organisms, predominantly fish pathogens (Combe & Gozlan 2018). However they can also infect amphibians, mammals, and a range of invertebrate phyla (Glockling et al. 2013).

Constituted as well by multicellular and unicellular organisms, part of the evolutionary success of **holomycotans** (the “fungal” lineage of Opisthokonta), resides in their ability to grow hyphae. These chitin-covered tubular multinucleated cells penetrate by brute force in the substrate aided by extracellular enzymes (Naranjo-Ortiz & Gabaldon 2019). A major pre-adaptation that combined to an absorptive mode of nutrition have facilitated repeated transitions to parasitism (Mendgen & Hahn 2002, McLaughlin et al. 2009) in every lineage (Fig. 4). Early evolving unicellular lineages such as aphelids, microsporidians, or chytrids, routinely classified as protists, also present parasitic lifestyles (Vávra & Lukeš 2013, Karpov et al. 2014). In spite of being the causative agent of a number of important infections in vertebrate and invertebrate animals (Fisher et al. 2012), holomycotans are, unlike holozoans, particularly pathogenic for other fungi, protists, plants, and algae (Maor & Shirasu 2005, Frenken et al. 2017).

Amoebozoa, the other major clade within Amorphea, groups seven distinct lineages of amoeboid protists (Fig. 4), three of which (archamoebae, discoseans, and tubulinids) are important parasites of animals (Schuster & Visvesvara 2004, Pawlowski & Burki 2009).

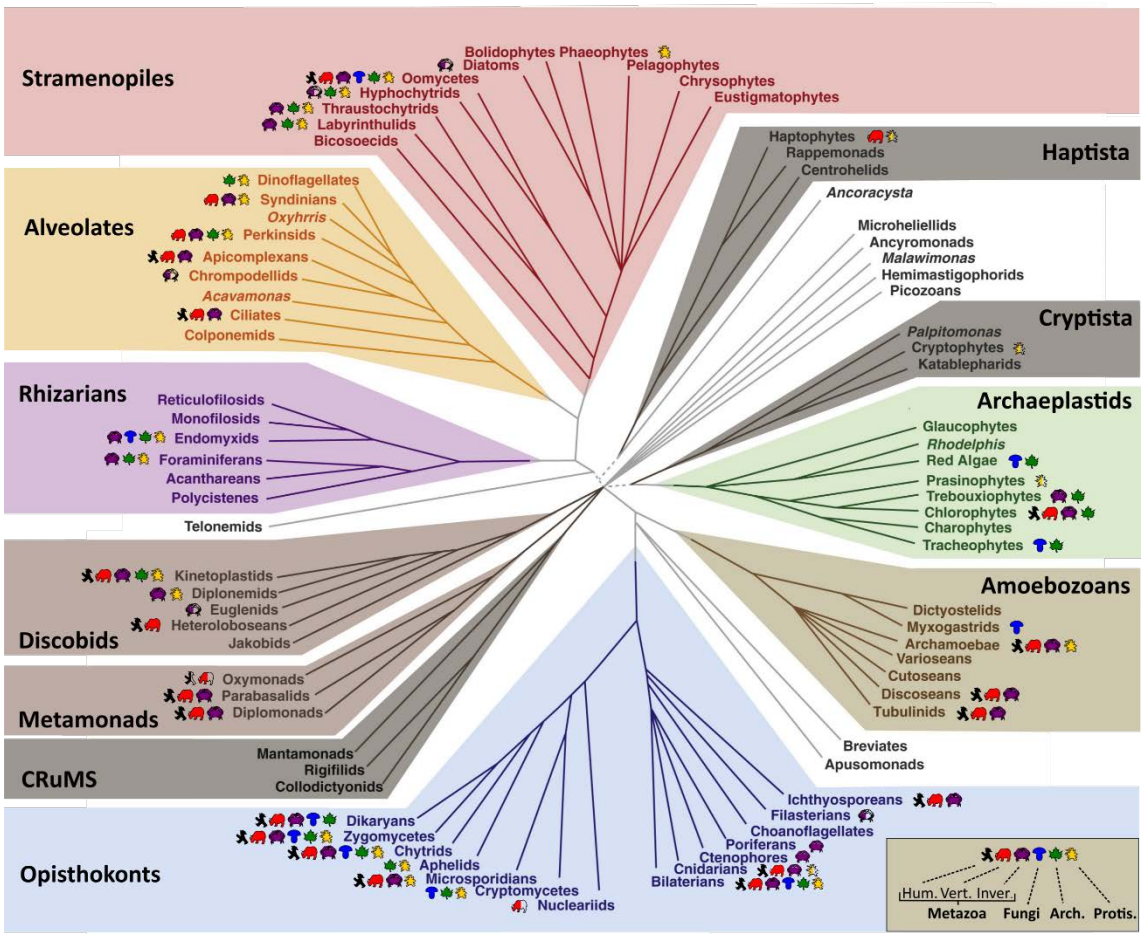

Figure 4. Lineages including parasitic taxa within the current phylogeny of eukaryotes. The baseline phylogenetic tree of eukaryotes has been adopted from Keeling & Burki 2019, and modified to review lineages including parasitic taxa. Shapes representing a human (black), a rhyno (red), a crab (purple), a mushroom (blue), a leaf (green), and an amoeba (yellow), indicate that at least a taxon contained within that clade is parasitic for humans, vertebrates, invertebrates, fungi, archaeplastids, or other protist lineages respectively. Half coloured shapes indicate a symbiotic association in which the antagonistic effect is non-existent or insufficiently documented.

Moreover, few myxogastrid genera like *Trichia*, *Metatrichia*, *Stemonitis*) can parasitize fungi (Ing 1994). Several amoebas, especially those belonging to Archamoebae, such as *Endolimax* sp., *Entamoeba* sp., or *Iodamoeba* sp., but also the discoseans *Saphinia* sp. and *Acanthamoeba* sp., or the tubulinids *Hartmannella* sp. and *Vermamoeba* sp., are parasitic in humans, becoming truly pathogenic in some cases (Visvesvara et al. 2007). For instance, infections caused by *Entamoeba* spp., refered to as amebiases, are amongst the most significant human infections, representing the third most common cause of death by parasitic diseases after Malaria and Schistosomiasis (Haque et al. 2003, Faust & Guillen 2012). In turn, amoebic gill disease (AGD), which is caused by the archamoebid *Neoparamoeba perurans* is responsible for recurrent pathologies and mortality events in farmed and wild salmonids (Adams & Nowak 2001, Rodger 2014). Understudied species within this supergroup have been observed to infect

invertebrates as well, including echinoderms, crustaceans, insects, or molluscs (Jones et al. 1985, Dyková et al. 2000).

Possibly, the presence of several parasites among plants, green/red algae, and their unicellular relatives (Supergroup **Archaeplastida**) is more surprising, especially for those lineages such as Trebouxiophytes and Chlorophytes which include taxa capable of infecting invertebrate and even vertebrate animals (Fig. 4; Osumi et al. 2008, Barsanti et al. 2008). For instance, the non-photosynthetic genus *Prototheca* is constituted by species capable of infecting fish, amphibians, cattle, and occasionally humans, causing protothecosis (Roesler et al. 2006, Jagielski et al. 2017). In turn, genera *Helicosporidium*, *Chlorella*, *Coccomyxa*, or *Elliptochloris* have only been superficially studied as parasites of invertebrates (Tartar & Boucias 2004, Crespo et al. 2009). In contrast, there are hundreds of examples of archaeplatids parasitizing other plants, algae, and even fungi (Goff et al. 1997, Brooks 2004, Selosse & Roy 2009), although they only represent around 1% of the described archaeplastid taxa (de Meeûs & Renaud 2002). Apparently, a significant fraction of the genes involved in the photosynthetic metabolism, if released from that selective pressure, could evolve with certain ease to allow a parasitic lifestyle once photosynthesis is lost (Oborník 2019). Although parasitizing another photosynthetic organism is considerably easier for archaeplastids, given the similarities between metabolic pathways (Woo et al. 2015); animal-infection by phototrops is hypothesized to have arisen again from opportunity, possibly from comparable symbioses such as the one existing with corals (Mohamed et al. 2018).

No evidence of parasitic lifestyle has been documented, to the best of our knowldge, in Supergroups **Cryptista**, **Haptista**, and **CRuMS** (Fig. 4), except for a putative coccolithophorid causing skin-associated disease in spiny dogfish *Squalus acanthias* (Leibovitz & Lebouitz 1985). Additionally, few haptophyte and cryptophyte taxa such as *Chrysochromulina andersoni* or *Teleaulax amphioxeia* are known to establish symbiotic associations to radiolarian and dinoflagellate protist lineages respectively (Janson 2004, Yamaguchi et al. 2011, Yuasa et al. 2019).

Quite the contrary occurs among excavates (Supergroups **Discoba**, **Metamonada**, and Malawimonada), which gather some of the better known and most concerning protist parasites, pathogens and diseases (Simpson et al. 2006, Manning et al. 2011, Bilbe 2015). Among **Metamonada** Grassé 1952, parabasalids and diplonemids are particularly relevant, as they include genera of medical and veterinary importance such as *Giardia* or *Trichomomas*, the causetive agents of giardiasis and trichomoniasis respectively (Schwebke & Burgess 2004,

Minetti et al. 2016). Characterized by high levels of host-specificity and a wide range of vertebrate hosts these and other metamonad species are interesting as ecologically relevant models of host-symbiont coevolution (Monis et al. 2009, Malik et al. 2011). Furthermore, several diplonemid genera such as *Octomitus*, *Hexamita* and *Retortamonas,* which are capable of infecting vertebrates (mammals, birds, amphibians, fish) and inverterbrates (insects and molluscs) are key to undertand zoonoses (Jones-Engel et al. 2004, Helmy et al. 2018). **Discoba** Simpson 2009 is a clade of flagellated protists representing the bulk of excavate organisms (Adl et al. 2019). While the majority of described species are free-living aquatic bacteriovores, the group also includes several pathogenic species of great concern, especially within Kinetoplastea Honigberg 1963. Apart from human infecting taxa such as *Leishmania* sp. or *Trypanosoma* sp., the lineage includes species with monoxenous (one host) and dixenous (two hosts) life cycles, which evidence a high degree of genetic and mophological pre-adapation to parasitism in this early evolving lineage (Yeo et al. 2005, Lukeš et al. 2014, Torres-Guerrero et al. 2017).

Hosting the greatest part of protists diversity and abundance, especially in aquatic ecosystems (de Vargas et al. 2015), lineages within the **TSAR** Supergroup are not especially concerning for human health, with the notorius exceptions of haemosporid and eimerid apicomplexans (*Plasmodium* spp. (Malaria), *Hepatocystis* spp., *Eimeria* spp., *Toxoplasma* sp.), and few ciliate and oomycete species (Fig. 4; Kamoun 2003, Schuster & Ramirez-Avila 2008). However, the Supergroup includes some of the most diverse and significant pathogens of invertebrates, plants, and other protists (Sierra et al. 2016); result of many independent transitions to parasitism and later diversifications in a highly divergent and abundant clade (Mathur et al. 2019). For instance, oomycetes have converged with fungi in many morphological and genetic adaptations to parasitism, including the formation of hyphae-like structures, host-penetration, or the development of genetic elements allowing rapid genome reorganization (Kemen & Jones 2012). In turn, the presence of an apical complex (a system of structures and organelles in the ápex of the cell) or equivalents in several alveolate lineages (Perkinsids, Syndinians, Apicomplexans) indicates certain morphological **pre-adaptation** to penetrate inside host cells (Okamoto & Keeling 2004). Besides, the endosymbiotic lifestyle of many ancestral apicomplexans represents elevated **opportunity** to transition to a parasitic lifestyle in the lineage (Leander 2008).

3. Invertebrate-infecting protist parasites

3.1. The role of invertebrates as hosts

The vast majority of animals in this planet are invertebrates, and accordingly, they possibly host most of the existing parasite diversity (Leung et al. 2015). Moreover, they lack an acquired immune system (senso strict), a very effective barrier against pathogens, that renders them, in principle, more vulnerable to parasitic infections (Anderson & May 1981, Loker et al. 2004). Furthermore, the emergence and diversification of several lineages of micro-eukaryote parasites preceded the vertebrate radiation (Kopečná et al. 2006); while many of them have evolved to infect vertebrates, others remain parasitic only for specific invertebrate groups (Burki & Keeling 2014).

However, what we know about invertebrate-infecting protist parasites, their host range, diversity, ecology, and distribution is still relatively limited in comparison to vertebrates (Marcogliese 2002). Several reasons exist for this apparent imbalance. Firstly, the reduced knowledge of the invertebrate host groups themselves, including biological, ecological, or bio-geographical data, readily available for most vertebrate lineages (Poulin & Morand 2000). A better understanding and comprehensive record of human, cattle, bird, and even fish population dynamics allow detecting unexpected alterations and investigating potential causative agents. Secondly, the historical lack of commercial interest in invertebrates, except for certain mollusc, crustacean, and insect species such as bees or silk-worms (Keeling 2009). Lastly, the traditional conception of parasites, especially micro-eukaryotes, as a “nuisance”, rather than a crucial part of food-webs has prevented their study and integration into the research done by fields other than parasitology (Lafferty et al. 2006, Dobson et al. 2008). Despite their crucial role maintaining diversity and controlling disproportionate insect, algal, or fungal growths (pests, blooms), only recently have a handful of parasite species been included in the International Union for Conservation of Nature, IUCN (Dougherty et al. 2016, Carlson et al. 2020). For most, it will still sound daft the need to study, not to say conserve micro-eukaryotic parasites infecting invertebrates.

Naturally, the research effort destined to investigate protists pathogens of humans and close or consumed vertebrates species (pets, cattle, birds, or fish) is much greater than the one conducted in invertebrate phyla. Especially in aquatic environments, where most host pathological surveys target fish and shellfish, rarely including other invertebrate groups (Rhode 1993, Stentiford & Feist 2005). In fact, among the invertebrate-infecting protist lineages, the

most diverse are Apicomplexa, Microsporidia, Kinetoplastea, and Myxozoa (Fig. 5), all comprising numerous species with heteroxeneous lifecycles (>1 host) capable of infecting humans or key vertebrate groups (Canning & Okamura 2004, Morrison 2009, Rodrigues et al. 2014, Stentiford et al. 2019). In turn, other very significant lineages of vertebrate-affecting pathogens such as Parabasalids, Diplonemids, or Archamoebae hardly include any known invertebrate host (Fig. 5), not necessarily meaning nonexistence, but rather unbalanced research (Keeling 2009, Stensvold et al. 2009).

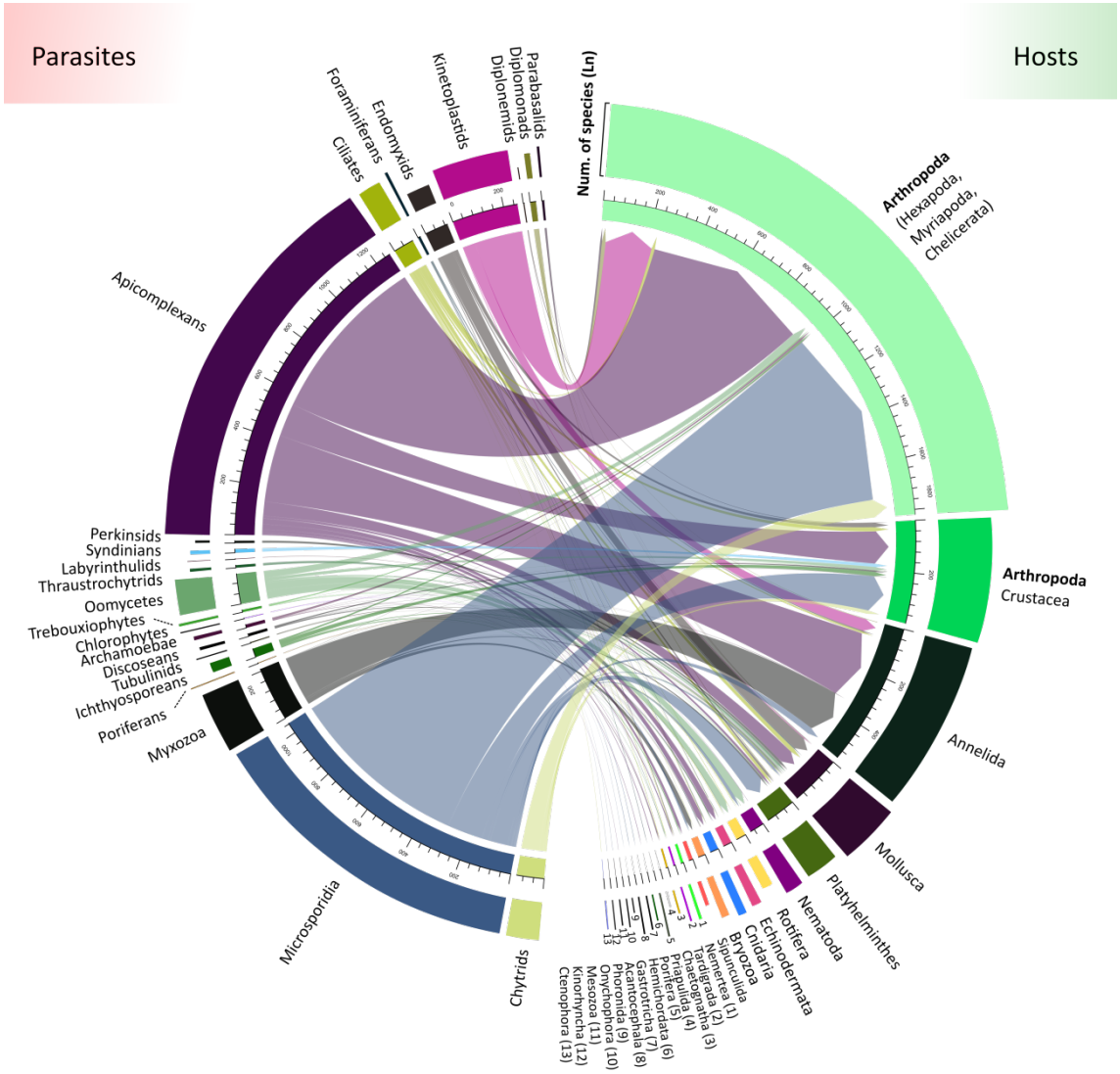

Figure 5: Summary of the invertebrate-infecting protist lineages (specified in Fig. 4) and their respective hosts. The width of the arrows is proportional to the number of parasite taxa in each lineage. For instance, there is 260 species of invertebrate-infecting myxozoans, of which 180 infect annelids. The outer circle indicates the number of described species (naepierian logarithm) in each of the parasitic and host clades. For instance Myxozoa is constituted by 2200 species and Annelida by 16.700 species.

An equitable understanding of the actual distribution of parasites and their environment (the host), represent an excellent and measurable model (molecular phylogeny) to test

evolutionary ideas regarding speciation and diversification among related taxa (Poulin 1999). The quick advance and democratization of DNA sequencing techniques has stepped up the discovery of cryptic diversity among many lineages of protist parasites in the marine environment (**Introduction - Section 4**). However, the study of their host-ranges, lifecycles and transmission routes is struggling to keep up the pace (**Introduction – Section 5**). In this context, understanding the ecology and spatiotemporal distribution of the parasitic micro-eukaryotes to which these sequences correspond (**Introduction - Section 6**) will largely depend on having sufficient morphological, structural, and histopathological insights from related species.

3.2. An overview of invertebrate-infecting protist lineages

In spite of including very significant human pathogens, parabasalids and diplomonads, the two main lineages constituting supergroup Metamonada are predominantly endosymbiotic among invertebrates (Malik et al. 2011). Comprising about 450 species, just a dozen of **parabasalids** have been shown to parasitize non-vertebrate hosts, including molluscs, annelids, and insects, especially termites (Kutisova et al. 2005). A similar host range is observed among parasitic genera of **diplomonads**, such as *Retortamonas*, *Chilomastix*, or *Hexamita*, which apart from insects, annelids, and molluscs (oysters), infect marine platyhelminths too (Sokolova & Overstreet 2020).

The main lineage of invertebrate-infecting parasites in supergroup Discoba is **Kinetoplastea** (Fig. 5), which comprises about 500 species, a third of which infect insects (Votýpka et al. 2010). Leeches (Phylum Annelida) also represent key hosts for kinetoplastids, hosting at least 50 different parasite species (Stevens 2008, Lukeš et al. 2014). Seldom, a handful of species from genera *Cryptobia* and *Icthyobodo* have been observed to cause infection in molluscs, including abalones and octopuses (Forsythe et al. 1991, Chen et al. 2000). Hyperdiverse in marine environments but almost uncharacterized (Lukeš et al. 2015), **Diplonemea** Cavalier-Smith 1993, a lineage sister to Kinetoplastea, includes free living and parasitic species (Fig. 5). So far, only two diplonemid genera are known to infect invertebrates; *Diplonema* and *Rhynchopus,* parasites of clams and lobsters respectively (Roy et al. 2007, Takishita et al. 2020).

The most significant lineage of rhizarian parasites (supergroup TSAR) is surely **Endomyxa** Cavalier-Smith 2002 (Fig. 5). The group is constituted by clades Vampyrellidea, Phytomyxea, Gromiidea, and Ascetosporea (Adl et al. 2019). While phytomyxids include important obligate parasites of plants, algae, and other protists (diatoms, oomycetes), all ascetosporeans are obligate parasites of invertebrates, especially molluscs (Stentiford et al. 2013, Hartikainen et al.

2014, Murúa et al. 2017). Several pathogens within this lineage, such as *Marteilia*, *Bonamia*, *Minchinia*, *Haplosporidium*, or Microcytos are responsible for great mortality events in commercial and wild bivalve populations worldwide (Bass et al. 2019). Moreover, a number of ascetosporean species have been observed to infect annelids, crustaceans, nemerteans, and platyhelminths too (Burreson & Ford 2004, Carballal et al. 2005, Ward et al. 2016). In contrast, only about a dozen species of **foraminiferans**, the other rhizarian lineage to include parasites of invertebrates (Walker et al. 2017), have been described in a group comprising over 10.000 species (Murray 2007).

Protist parasites within clade Alveolata (Supergroup TSAR) are amongst the most significant and diverse to infect invertebrate animals (Fig. 5). This group includes several species-rich lineages of predominantly obligate symbionts and parasites, in which their mutualistic-parasitic continuum is not always clear-cut (Dziallas et al. 2012, Rueckert et al. 2019). For instance, with around 4500 species, **ciliates** are usually ecto- and endo-commensals, appearing associated to fundamentally all invertebrate phyla (Weisse 2017). However, parasitic species have also been described, particularly among molluscs, arthropods, platyhelminths, and annelids; although ctenophorans, rotiferans, nemerteans, echinoderms, or tardigrades can be parasitized as well (Morado & Small 1995, Vecchi et al. 2016, Peters 2021). In turn, **Apicomplexa** Levine 1980 is possibly the largest group of obligate endoparasites infecting invertebrates (Morrison 2009). The clade is constituted by about 6000 described species divided in four main lineages: gregarines, coccidians, haemosporids, and piroplasmids (Rueckert et al. 2011, Levine 2018). All four include important parasites of vertebrates and invertebrates such as *Plasmodium* sp., *Theileria* sp., *Eimeria* sp., or *Hepatozoon* sp. (Upton et al. 1990, Smith & Desser 1997, Criado-Fornelio et al. 2003). Affecting all major invertebrate taxa, they occur principally among arthropods and annelids (Fig. 5), habitual intermediate and reservoir hosts (Schnittger et al. 2012, Oborník 2020). In contrast, **perkinsids** and **syndinians**, the other two lineages of invertebrate-infecting alveolates, include very significant pathogens of bivalves and crustaceans respectively (Murrell et al. 2002, Small 2012).

Oomycetes represent certainly the most diverse and noteworthy lineage of parasitic Stramenopiles Patterson 1989 (TSAR supergroup) infecting both vertebrates and invertebrates (Bruno et al. 2011). Owing to a predominantly saprophytic lifestyle, and the formation of hyphae-like specialized structures (haustoria), they have been traditionally included within Fungi (Beakes et al. 2012). Over a tenth of the 1,200 species described in this group are pathogenic for invertebrates (Fig. 5; Thines 2018) especially rotifers, crustaceans, and molluscs; but also for less frequently surveyed invertebrate phyla such as Nematoda, Bryozoa, Sipunculida, or Tardigrada

(Beakes & Sekimoto 2009, West & Beakes 2014, Spies et al. 2016). In comparison, the other two lineages of Stramenopiles capable of parasitizing invertebrates, **Labyrinthulida** and **Thraustrochytrida**, are almost exclusively found in molluscs (Azevedo & Corral 1997, Stokes et al. 2002, Schärer et al. 2007, Burge et al. 2013).

Among species within Supergroup Archaeplastida Adl et al. 2005, hundreds parasitize other plants, algae, and even fungi (de Meeûs & Renaud 2002, Press & Phoenix 2005), but only few unicellular **Trebouxiophytes** and **Chlorophytes** infect invertebrate animals (Fig. 5). Several species from genera *Helicosporidium* and *Chlorella* parasitize arthropods (insects, mites, springtails, or cladocerans), nematodes, and platyhelminths (Tartar et al. 2002, Pombert et al. 2014). In turn *Coccomyxa* spp. are parasitic for bivalves end echinoderms (Rodríguez et al. 2008, Crespo et al. 2009). At least two genera, *Ellipthocholoris* and *Entocladia*, have been proposed to be in the parasitic side of the symbiotic spectrum when associated to cnidarians (Goldberg et al. 1984, Gustavs et al. 2017).

Despite their significance as parasites of humans and other vertebrates (Schuster & Visvesvara 2004, Nowak et al. 2014), only a handful of protists in supergroup Amoebozoa Lühe 1913 are known to infect invertebrates (Fig. 5). Arthropods represent the main hosts for **archamoebid amoeba**, which are also facultative parasites of chaetognaths, and annelids (Dyková et al. 2008, Constenla et al. 2014). Essentially the same host range is observed among parasites in lineage **Discosea** Cavalier-Smith 2004, which are especially prevalent among crustaceans (Han 2019), but also capable of parasitizing echinoderms (Nowak & Archibald 2018). Conversely, its sister lineage, **Tubulinea** Smirnov et al. 2005, the richest of these invertebrate-infecting ameboid lineages in number of species, only parasitize few molluscs and a cnidarian species (Hertel et al. 2002, Maxwell 1970).

Finally, among the protist or protist-considered lineages constituting supergroup Opisthokonta Cavalier-Smith 1987, the most significant ones to infect invertebrates are **Myxozoa** Grassé 1970 and, above all, **Microsporidia** Balbiani 1882 (Fig. 5). Constituted by obligate intracellular parasites (Feist & Longshaw 2006), myxozoans are particularly prevalent among annelids and bryozoans, which act as intermediate hosts for several vertebrate-infecting pathogens (Holzer et al. 2018). Besides, these microscopic cnidarians have also been reported to parasitize arthropod, mollusc, and platyhelminth species (Yokohama & Masuda 2001, Sokolova & Overstreet 2020). Obligate intracellular parasites too, the range of invertebrate hosts infected by microsporidians is considerably larger (Fig. 5). Although particularly common among insects, crustaceans, and platyhelminths (Becnel & Andreadis 1999, Stentiford et al.

2013), microsporidian parasites have been described parasitizing organisms in essentially every invertebrate phylum (Keeling & Fast 2002, Mathis et al. 2005). Noticeably smaller, opisthokont lineages **Ichthyosporea** Cavalier-Smith 1998 and **Chytridiomycota** Doweld 2001 still comprise several dozen invertebrate-infecting parasites, especially of arthropods (Glockling 2013). Molluscs, nematodes, rotifers, echinoderms, sipunculids, or tardigrades have also been shown to be potential hosts (Dewell et al. 1985, James et al. 2006, Orpin 2020).

4. Hidden diversity among invertebrate-infecting protist lineages

The unremitting expansion of DNA sequencing methods, bioinformatic tools, and computing resources is transforming our understanding of protistan communities and diversity (Caron & Hu 2019, Giner et al. 2020, Holman et al. 2021). This standardization of molecular-based research is allowing protistologist to penetrate into a world largely restricted due to microscopic size, quick interactions, and ambiguous taxonomic relationships (Gao et al. 2016). Accordingly, the significance of the role played by micro-eukaryotes in aquatic ecosystems as autotrophs, heterotrophs (predators, decomposers, parasites) and myxotrophs appears to be increasing by the day (Zubkov & Tarran 2008, Mitra et al. 2014, Bjorbækmo et al. 2020).

While ever larger multi-gene phylogenies are proving invaluable to trace the evolutionary history of protist lineages and Supergroups (**Introduction- Section 1**), molecular studies based on short DNA sequences are opening a "Pandora´s box" of micro-eukaryotic diversity (Keeling & Del Campo 2017). Broadly targeted gene amplicon studies, particularly those using the highly conserved 18S SSU rRNA, are showing substantial genetic divergence between morphologically similar species (Bass et al. 2015, Singer et al. 2018). The discovery of these so-called cryptic species goes beyond the escalation in biodiversity; it affects studies on cell-biology, ecology, lifecycle, and spatiotemporal distribution (Epstein & López-García 2008, Poulin 2014, Oliverio et al. 2020).

The unearthing of cryptic species is especially evident among parasitic protists, which on top of the limited morphological features already characterizing the clade, often experience a further reduction resulting from adaptation to life-conditions inside the hosts (Barta 2001, Detwiler et al. 2010, Perkins et al. 2011). Moreover, evolving under similar evolutionary pressures, phylogenetically diverse parasites show high levels of morphological conservation and convergence (Pérez-Losada et al. 2009, Poulin 2011). In consequence, traditional microscopy-based identifications and descriptions, which are frequently conducted on resistant forms (spores, cysts) and stationary developing stages in histological preparations, have

underestimated the actual diversity of parasites (Criscione et al. 2005, Bass et al. 2015). For instance, the number of *Plasmodium* spp. causing avian malaria increased from 175 species identified via morphology to almost 10,000 when DNA-based analyses were applied (Bensch et al. 2004, Martinsen et al. 2008). This represents a good example of how the discovery of cryptic species affects estimates of host specificity. What based on microscopy was thought to be a single parasite infecting few host species, turns out to represent a complex of cryptic species each specific to a single host (Poulin & Keeney 2008).

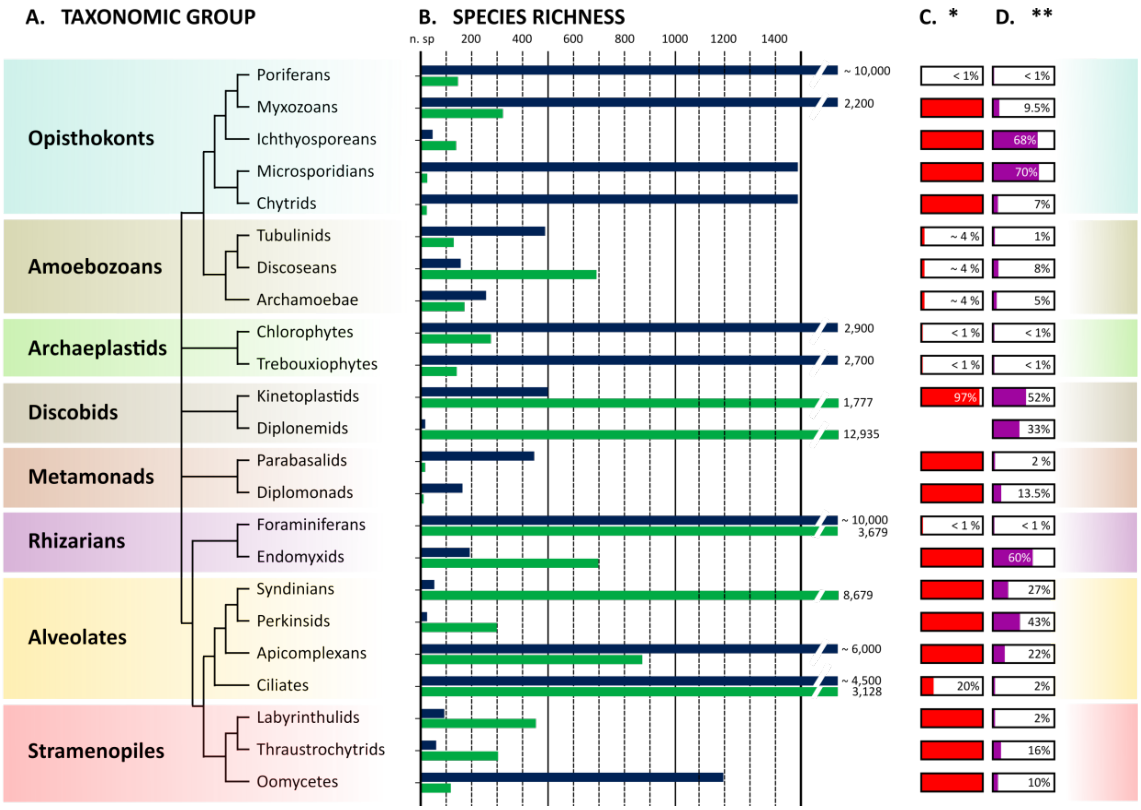

Figure 6. (A & **B)** Graph comparing the current number of species in every invertebrate-infecting lineage (blue bar) and the species richness (OTUs) discovered by the Tara Oceans expedition (de Vargas et al. 2015) with an expansion to the deep sea by Schoenle et al. 2021 (green bar). **(C)** Ratio indicating the percentage of species within each clade described as parasites by de Meeûs & Renaud 2002. **(D)** Ratio representing the number of invertebrate-infecting parasite species from the total number of species in each clade, calculated from review in (Fig. 5).

In addition to the apparent imbalance between morphological and genetic methods to detect diversity, parasitic lifestyles seem to promote a rapid sympatric speciation (Perkins et al. 2011). The rationale behind is that both, host individual and population, represent a continuously changing environment and breeding site, in which the number of diversifying factors is potentially larger for parasites than for their free living counterparts (Huyse et al. 2005, Forbes et al. 2009). As a result, the cumulative curve of cryptic species being discovered for most protist parasite taxa is still rising steeply or is only beginning to show a slow down (Appeltans et

al. 2012, Poulin 2014, Caron & Hu 2019). Whether this hidden diversity is mounting as a corollary of greater discrimination means or increased speciation pace among parasites, it is definitely influencing our understanding of host specificity and with it our knowledge of parasite lifecycles, prevalence or distribution (Jousson et al. 2000, Škaloud et al. 2019).

Early molecular-based detection and identification of micro-eukaryotic parasites relied on PCR screenings of infected or suspected hosts using species-specific primers (Kimura et al. 1997, Whipps et al. 2003). This methodology is easily combined with visual techniques such as microscopy, still a tool of paramount importance for clinical diagnostics (Paoletti et al. 2018, Kerr et al. 2018, Helmer et al. 2020). Furthermore, the use in parallel of molecular methods and microscopy is crucial to link genotypic diversity to phenotype, including morphological, ultrastructural, pathological, and behavioural observations (Bjorbækmo et al. 2020). However, over the last 15 years the consolidation of Next Generation Sequencing (NGS) techniques has, in some way, inverted this course (microscopy -> molecular) of action (Valkiūnas et al. 2008, Certad et al. 2019, Santoferrara 2019). Now, using methods such as High Throughput Sequencing (HTS), billions of DNA or RNA sequences are generated from a single sample taken in the environment (environmental DNA, hereafter "eDNA") or from an organismal matrix, ahead or without microscopical examination (Bass et al. 2015, Massana et al. 2015, Mukherjee et al. 2019). The flourishing of such approaches has roqueted the discovery of genetic diversity within many protists lineages, not only in the environment but also associated to specific hosts or even tissues (Bass et al. 2019). Inexorably, this strength displayed by DNA sequencing and analysis methods is opening up the gap between genetic diversity and our understanding of the ultrastructure, ecology, pathology, or host-range of the "putative" parasites constituting it (Ward et al. 2018, Käse et al. 2021). As discussed by Keeling & Del Campo (2017), understanding the extent of what we don´t know is highly important to see where efforts and combined approaches should be applied.

Among the protists lineages shown to include species capable of parasitizing invertebrate hosts **(Introduction – Sections 2** & **3)** several clades appear to contain a great deal of undiscovered diversity (Fig. 6). The figure represents the actual number of described species for every lineage (references in annex 1) and the ratio of parasites/free living stages measured in each (reviewed by de Meeûs & Renaud 2002). This approximation to the current number of parasite species per lineage is compared to the estimated species-richness (expressed as number of OTUs) registered in the extended Tara Oceans global metabarcoding dataset (de Vargas et al. 2015, Schoenle et al. 2021). As OTUs (Operational Taxonomic Units) are only a proxy for species, and not species (Mysara et al. 2017), sequences from independent metabarcoding

surveys should be merged and phylogentically analysed to estimate the real hidden diversity in each clade. Therefore, it is important to stress that the graph (Fig. 6) does not represent total exiting genotypic diversity within a clade, but only the findings by a single environmental study, although possibly the most complete one to the date (Gorsky et al. 2019). Additionally, the graph also reflects the number of invertebrate-infecting species within each lineage (invertebrate-infecting species / total species) using data from my own review in (Fig. 5). In this fashion, extrapolation of this (minimum) hidden diversity to the ratio o both total parasites and invertebrate-infecting parasites can give us an idea of the potential number of undiscovered parasites in each protists lineage, allowing to identify specially concerning ones.

Protists within supergroup Discoba represent a good example (Fig. 6). The number of putative **kinetoplastid** species is at least three times greater than described. The clade which includes significant pathogens of humans such as *Leishmania* sp., or *Trypanosoma* sp. (Dedet & Pratlong 2000), often has insects and leeches as vectors, reservoirs, or intermediate hosts (Simpson et al. 2006). Moreover, the clade also includes species capable of infecting fish and molluscs (Fig. 5), which being less studied than humans and key vertebrate species will likely host a significant fraction of that hidden diversity (Vickerman 1994). Identifying accurately which genotypes infect vertebrates, invertebrates, or both, in complex life cycles will provide fundamental information about parasite-host coevolution and distribution, highly valuable insights for epidemiology and epizootiology (the study of disease transmission between wildlife and humans). In the case of **diplonemids**, the other parasitic lineage within Discoba, the difference between described and putative species is extraodinary. Currently constituted by 12 described species (Flegontova et al. 2016) four of which infect clams and lobsters (Roy et al. 2007, Takishita et al. 2020), there is at least 13.000 cryptic species (Fig. 6). Thus, the clade, one of the most diverse and less known of all protist lineages (Lukěs et al. 2015) might horde great hidden diversity of micro-eukaryotic parasites, possibly parasitizing molluscs and crustaceans.

The extent of undiscovered diversity appears to be enormously high among alveolate lineages **Syndiniales** Loeblich 1976 and **Perkinsidae** Levine 1978 as well. The first includes few but very significant species of crustacean-infecting parasites, such as *Hematodinium* sp, the causative agent of the “bitter crab” disease (Stentiford et al. 2001). This highly lethal infection affects over 30 different decapod species, some of them with great commercial value (Stentiford & Shields 2005). The clade also includes genus *Amoebophyra*, which groups several species of dinoflagellate-infecting parasites that play a crucial role in the ecosystem, controlling the excessive growth algal blooms (Kim 2006, Cay et al. 2020). A much better comprehension of the syndinid host–parasite associations and lifecycles, which are not even fully resolved for the

better studied species (Xu et al. 2010), will be of paramount importance to contextualize the almost 9000 cryptic species discovered just by the Tara Oceans survey (de Vargas et al 2015). Cryptic diversity among **perkinsids**, a clade in which 43% of the described species infect invertebrates (all of them bivalves), has grown significantly too, passing from 21 described to over 300 putative species (Fig. 6). The most significant invertebrate-infecting genus within this clade is *Perkinsus*, which infects oysters, clams, and abalones globally (Lohan et al. 2018), causing very significant losses for the aquaculture industry (Casas & Villalba 2012, Pretto et al. 2014). Furthermore, in consonance with syndinians, several species within this lineage are also parasitic for harmfull bloom-causing dinoflagellates (Lepelletier et al. 2014), and possibly a wide range of fish species (Freeman et al. 2017, Gleason et al. 2019); inviting to attain a more comprehensive understanding of the cryptic diversity within the clade.

Among rhizarians it is particularly concerning the level of hidden **endomyxid** diversity, which sees the number of species almost quadrupled (Fig. 6) in a clade in which a 60% of the 200 species described are invertebrate-infecting parasites. The lineage comprises predominantly pathogens of molluscs, many of them with great commercial and ecological value (Le Roux et al. 2004, Carnegie et al. 2006, Hartikainen et al. 2014). This explains the renewed surge on the interest for the evolution and diversity of the clade (Ward et al. 2016, Sierra et al. 2016, Bass et al. 2019, Hittorf et al. 2020). The complex and largely unsolved lifecycles (including planktonic hosts) of some key commercial species (Audemard et al. 2001, Carrasco et al. 2008), highlight the importance of studying small invertebrates to untangle the many hidden associations occurring in the lineage (Arzul et al. 2014). Also within the TSAR Supergroup, the alveolate lineages **Labyrinthulida** and **Thraustochytrida**, in which the few invertebrate-infecting species (Fig. 5) are almost exclusively parasites of molluscs as well (Stokes et al. 2002, Schärer et al. 2007), would see the number of species quadrupled (Fig. 6). However, in these two groups, most cryptic species are likely parasites of plants and other protists lineages (Fig. 4), their principal hosts (Moro et al. 2003, Ueda et al. 2015, Marchan et al. 2018).

Finally, and contrasting with the pattern observed in most of the lineages reviewed (Fig. 6), the number of OTUs detected by the “Tara Ocean” and “Deep Sea” expeditions (de Vargas et al. 2015, Schoenle et al. 2021) is considerably smaller than the number of existing species in lineages Oomycetes, Parabasalida, Diplomonads, Microsporidia and Chytridiomycota. It is well known that certain obligate endoparasites such as apicomplexans (excluding gregarines) and **microsporidians** are largely missing from broadly-targeted metabarcoding surveys (Dubuffet et al. 2021). Many parasites have highly-divergent SSU rRNA sequences that hold back amplification and resistant spores (**microsporidians**, **oomycetes**, and **chytrids**) that can impede

DNA extraction (Bass et al. 2015). Highly divergent and capable of forming cysts as well, the low detection of **parabasalids** and **diplomonads** (at least in the Tara Oceans Survey), is further explained by their endemicity. Besides, targeting the intestines of vertebrates and invertebrates, they are exposed to extra signal-reducing noise and acidic environments (Babaei et al. 2011, Tai et al. 2015). In such cases, the use of specific primers and special DNA extraction protocols is needed to conduct efective eDNA screenings (Bass et al. 2015, Beng & Corlett 2020). However, the design of adequate primer sets obliges for a reasonable understanding of within-clade diversity first (Hartikainen et al. 2014), recalling a bit a dog chasing its own tail. This evidences the value of parallel microscopical examinations of environmental or organismal matrixes.

5. Lifecycles and transmission strategies of protist parasites infecting invertebrates

Recent ecological models set to understand biological interactions and energy flows in the ecosystem have seen far-reaching variations after considering parasitic links (Lafferty et al. 2008, Dunne et al. 2013, Preston et al. 2014). However, there are two major obstacles hampering a more widespread inclusion of parasites into ecological and food-web models. The first limitation is the food-web theory itself, which is struggling to accommodate parasitic interactions after passing over them since its inception (Marcogliese & Cone 1997, Byers 2009, Jephcott et al. 2016). The second obstacle is that a vast majority of those links (parasite-host associations) remain undiscovered, especially for invertebrate hosts (**Introduction – Section 4**). Many parasites have complex life cycles involving different hosts and cell forms, but excepting the main host(s) for human concern, these remain poorly comprehended (Bass et al. 2015, Okamura 2016).

Currently, one of the key goals for parasitology stays to integrate traditional methods to elucidate life cycles (experimental infections, microscopy) with the same molecular approaches that have allowed the unearthing of such a vast hidden diversity (Nadler & Pérez-Ponce de León 2011, Blasco-Costa & Poulin 2017, Keeling 2019). A sound and balanced knowledge of the lifecycle of parasites, including the recognition of infective forms, vectors, reservoirs, or dormant/resistant stages, can be determinant to understand when and where infection pressure on the host and the community might vary (Pickles et al. 2013, Bass et al. 2015). Such ontogenic insights will allow not only developing more realistic and complete ecological models, but also conducting essential surveillance and predictive epidemiological and aetiological analyses (Thompson & McManus 2001). These, in turn, consent the design of control programs

or treatments for those diseases of special concern (King 2010, Lorenz & Koella 2011, Suarez et al. 2017, Herren et al. 2020)

Here, the type lifecycles and transmission strategies of the main invertebrate-infecting protist parasite lineages will be reviewed, but just as it has been done with the concepts of symbiosis or parasitism, some key concepts will be defined first. Many micro-eukaryotic symbionts have a single host during their lifecycle, spending at least some time outside the host, this are the so called **monoxenous** parasites. Others have two and occasionally even more hosts (**heteroxenous** parasites), often belonging to widely separated taxonomic groups (Loehle 1995, Kreier 2013). In the case of parasites with heteroxenous (= complex, indirect) lifecycles, the hosts are distinguished from each other, as intermediate and definitive hosts (Parker et al. 2015). The **intermediate host** is that in which the parasite undergoes some level of development, but does not reach sexual maturity. The **definitive host** is the one in which the parasite is able to sexually reproduce (Criscione et al. 2005).

However, as it occurred between commensalism and parasitism, the difference between them is not always clear-cut. For example, in those organisms where sexual reproduction does not occur, or has not been described yet, as it the case of certain kinetoplastids (Weedall & Hall 2015, Berry et al. 2019). Or among those parasites transitioning between monoxenous and heteroxenous lifecycles, in which the sexual reproduction commences in one and is completed in the other (Raugh et al. 2005). Furthermore, **paratenic hosts** are commonly recognized in the lifecycles of protist parasites. While in these associations the parasite does not develop inside the host, the latter is key to bridge an ecological gap in the parasite´s life cycle (Donoghue 2017). Such is the case of many erythrocyte-infecting parasites of vertebrates transmitted by blood-sucking invertebrates (Davies & Johnston 2000).

Additionally, it is also recurrent the concept of **reservoir host**, in which a parasite can survive and reproduce (asexually or sexually), but it is not considered the normal host (Bush et al. 2001). However, it is apparent, that the notion of “normal” host is anthropocentrically biased. To circumvent these conflicts, it has become widespread the use of **vector**, a controversial term to define a host that transmits an infectious agent to another host (review by Wilson et al. 2017). As discussed by Kreier (2013), in the practice, the term vector is often used to refer to the invertebrate host in complex life cycles in which the other host is a vertebrate or a plant. If the parasite grows and/or reproduces inside the vector, it is considered a **cyclical vector**; if neither growth nor reproduction occurs in the vector, it is referred to as a **mechanical vector**.

The confrontation between some of these concepts is derived from the complexity and uniqueness in the transmission strategy and lifecycle of many eukaryotic parasites (Goater et al. 2014). This miscellany of different parasite-host associations is the result, among others, of the numerous independent transitions observed in most eukaryotic lineages and supergroups, as already reviewed (**Introduction - Section 2**). In consequence, while profoundly influenced by phylogeny, the development of some mathematical models to analyse parasite-host population dynamics have been designed considering non-phylogenetic categorizations of parasitic lifecycles (Thomas et al. 2002, Lafferty et al. 2006). The most famous of these non-phylogenetic divisions, divides eukaryotic parasites in **macroparasites** and **microparasites** (Anderson & May 1992). This traditional and still widespread dichotomous categorization (Keeling & Rohani 2011) splits between those parasitic organisms in which virulence is not dependant on the number of infection events (microparasites) and those for which it is (macroparasites). The dependency is contingent on the ability of the parasite to **reproduce or not inside the host**, not on the host taxonomy or size (Poulin 2011). However, in the practice, most metazoan parasites end up being categorized as macroparasites and protists as microparasites (Morand et al. 2006).

The revolution in ecological modelling and food web-dynamics promoted by the enormous cryptic micro-eukaryotic diversity revealed by DNA since the beginning of the century, has resulted in a major revision to the traditional categorization of parasites. Using a factorial application of four dichotomies (number of hosts, virulence, intensity-dependency, transmission), each describing an essential biological aspect of the parasite-host interaction, Kuris and Lafferty (2000, 2002) recognized a wider suit of parasite categories. This categorization scheme on the parasitic strategies adopted by eukaryotes was largely maintained in a later major revision (Poulin 2011, Poulin & Randhawa 2013) and is increasingly used (O'Brien & Van Wyk 2017, Buck et al. 2018, Behringer et al. 2020). It distinguishes between six main strategies: parasitoids, parasitic castrators, directly-transmitted parasites, trophically-transmitted parasites, vector-transmitted parasites, and micropredators (Fig. 7).

Predominantly constituted by insects and certain fungi, **parasitoids** grow to a relatively large size inside their host, which must die for the parasite to emerge after completion of its life cycle. As a result of this high level of virulence parasitoids typically occur at low prevalence and intensity. Otherwise similar to parasitoids, **parasitic castrators** cause suppression (mechanical or enzymatic) of the host reproduction early during their development, deriving that energy for their own growth.

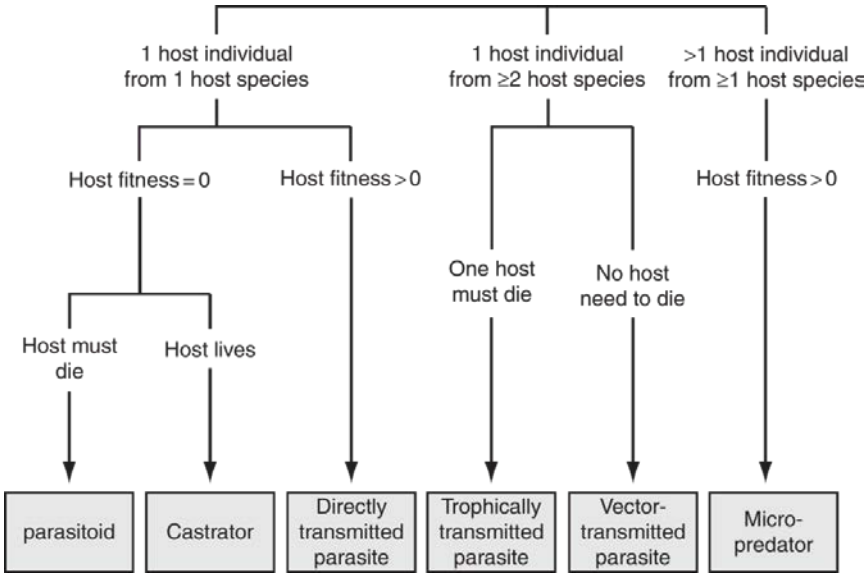

Figure 7: Classification tree of the six parasitic strategies considered by Poulin (2011), which encompasses the vast majority of known eukaryote parasite taxa. The first division is based on the number of hosts used, both in terms of species and individuals, by one full parasite generation; subsequent divisions are based on fitness impact on hosts.

Although the host survives, its inability to leave descent approximates its fitness to zero, and consequently their prevalence in the population must be low as well. Capable of reproducing in/on the host, **directly transmitted parasites** induce variable but intensity-dependant pathology. Including many species from invertebrate-infecting protist lineages such as Microsporidia, Amoebozoa, Apicomplexa, Diplomonada, Chlorophyta, Trebouxiophyta, Ciliophora, Endomyxa, or Ichthyosporea), they don´t generally kill the host as they are able to emerge as cysts, eggs, or spores, although they might be quite virulent (Fig. 8). Direct transmission is probably the first strategy adopted by any taxon transitioning from free living to parasitic, an almost inevitable pre-condition to evolve towards a heteroxenous lifecycle (Poulin & Randhawa 2013).

Requiring two or more different host taxa to complete their reproductive cycle, **trophically transmitted parasites** are habitual among myxozoans, microsporidians, apicomplexans, or parabasalids, (Fig. 8). In their case, virulence is usually higher in the intermediate host, as a way to increase the chances of being predated by the definitive host (Dunn & Smith 2001, Britton & Andreou 2016). Numerous species of apicomplexans, parabasalids, kinetoplastids, and fungi that require a second host for the completion of their life cycle are considered to be vector-transmitted parasites. In this case, the vector is usually a micro-predator and the definitive host a vertebrate, which sustains a significant part of the parasite´s replication and as a result high virulence levels (Costa et al. 2018).

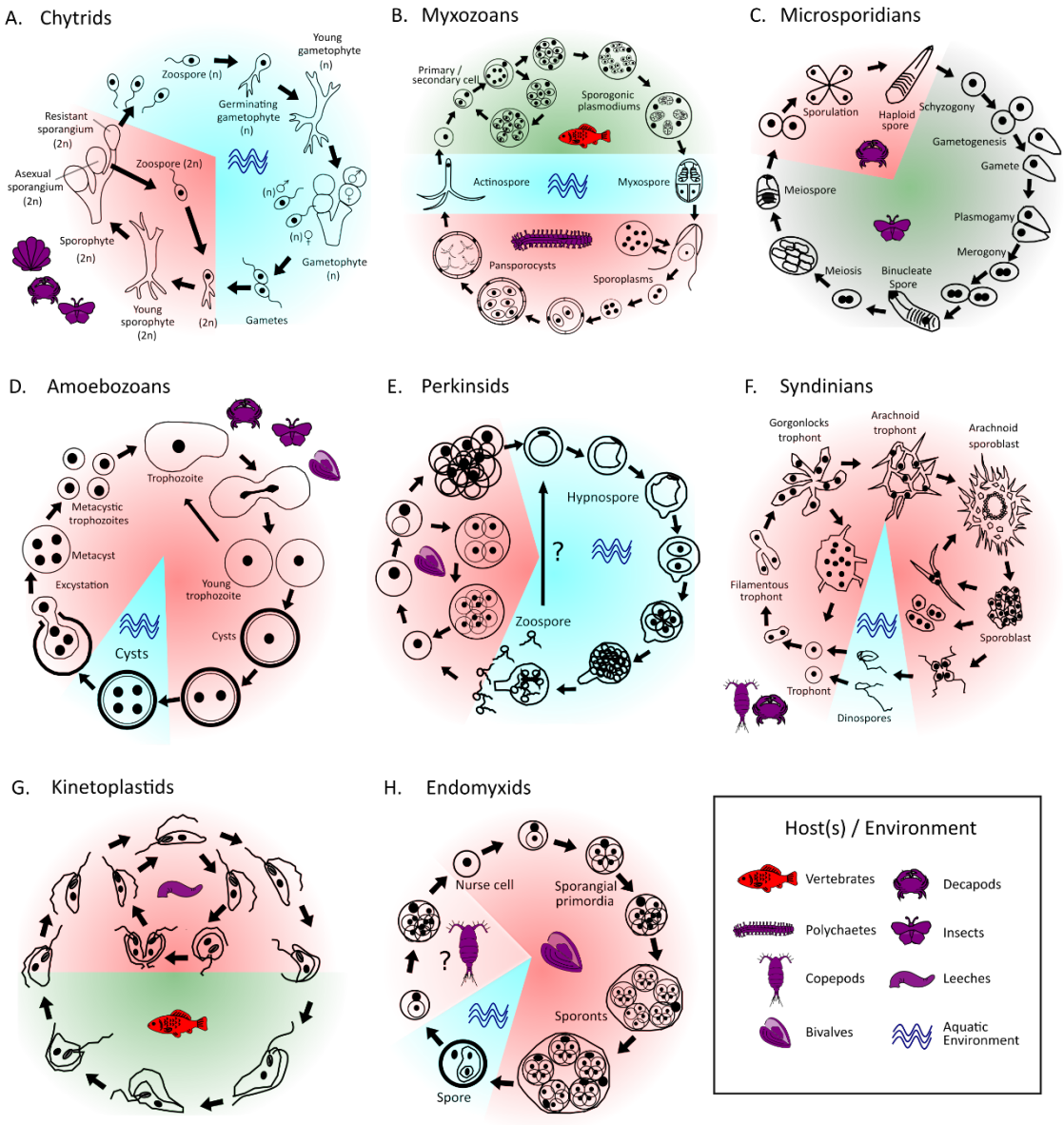

Figure 8: Generalized lifecycles of some major protist parasites from invertebrate-infecting lineages **(A)** Chytrids, are facultative parasites infecting mainly insects and crustaceans during their sporophytic lifecycle stage (modified from Raven et al. 1992). **(B)** Myxozoans are significant parasites of vertebrates, including fish, with polychaetes and bryozoans as main intermediate hosts (modified from Yokoyama et al. 2012). **(C)** Numerous microsporidian species have heteroxenous lifecycles with insects and crustaceans as hosts (modified from Andreadis 2007). **(D)** Several amoebozoan species reproduce in invertebrate hosts forming resistant cysts before leaving the host. **(E)** Perkinsids are exclusive parasites of bivalves in which their trophonts undergo vegetative multiplication before continuing a proliferative stage in seawater (adapted from Fernandez-Robledo et al. 2018). **(F)** Syndinians are known to infect decapods and copepods, usually within monoxenous lifecycles (adapted from Stentiford & Shields 2005). **(G)** Kinetoplastids genera such as *Cryptobia* have heterocenous life cycles in which leeches behave as intermediate hosts (modified from Melhorm 2008). **(H)** Endomyxids are significant pathogens of bivalves with small crustaceans behaving as posible reservoirs (modified from Perkins 1976 and Carrasco et al. 2015)

Lastly, **micropredation** constitutes the only strategy in which the parasite feeds on multiple host individuals from the same or different species during the same generation of their lifecycle (Fig. 7). The association is usually brief, lasting from few seconds to some weeks; usually with little cost to the hosts, unless the micropredator is vectorizing a more virulent parasite (ref). Several ciliate lineages and few early apicomplexans have been shown to have micropredatory-type strategies (Schotte et al. 2009, Gómez-Gutiérrez et al. 2017).

Noticeably, most protist lineages comprise taxa that fall within different parasitic categories, reflecting the broad continuum in trophic strategies existing in nature. This challenges assumptions about where one type of parasite-host interaction ends and another begins (Parmentier & Michel 2013). Parasitism, is the result of many independent transitions and posterior diversification events; even rebounds to free-living lifestyles (Klimov & OConnor 2013, Poulin & Randhawa 2013, Lukeš et al. 2014). These major parasite-adopted strategies represent **adaptive peaks** towards which, organisms jumping to a parasitic lifestyle, tend to converge (Poulin 2011). The parallelism among phenotypes (ultrastructure, behaviour, virulence) may occur simply because of genotypic and developmental constriction; or as a result of morphological adaptation under similar evolutionary pressures within a highly-specific environment, the host (Poulin 2014). Consequently, while infection, transmission, and reproduction strategies in a parasite species are profoundly influenced by genes and reflected by phylogeny, different adaptative endpoints, even within the same lineage often arise (Brown et al. 2001, Kuris 2003, Jackson 2015). It is imperative to understand well the contribution of genetic and/or morphological factors within each taxon and parasitic clade, in order to better estimate the potential role in the environment of the enormous cryptic diversity discovered.

6. Spatiotemporal variability of protist parasites

Unlike for multicellular organisms, the spatial and temporal distribution of most unicellular eukaryote taxa remains a profound mystery (Bråte et al. 2010, Alterrmatt et al. 2015, Oliverio et al. 2020). Think about mammals, amphibians, birds, or fish species; and also about plants, mushrooms, or multicellular algae. For all of them, a more or less complete record of their global distribution exists (Courtecuisse & Duhem 1995, Zhang et al. 2008, Parravicini et al. 2013, Ficetola et al. 2014, Stephens et al. 2016). After all, ecologist and often the wider public have studied the biogeographical and temporal variability of most of these lineages for centuries. In contrast, consider now micro-eukaryotes, it would be difficult to find more than a handful of examples in the literature apart from major diseases of humans such as Malaria, or *Trypanosomiasis*, in which the geographical and temporal distribution is reasonably well

delimited (Hay et al. 2004, Aregawi et al. 2019). This is only but normal, as we can only track the spatial and temporal shifts of those organisms that we are able to **observe** and **identify**.

As above discussed (**Introduction - Section 1**), the classification and taxonomy of protists has been considerably blurry and unstable, at least in comparison to that of the rest of eukaryotes (Adl et al. 2007, Bass et al. 2009). This has rendered even more challenging the identification of these often morphologically undistinguishable organisms (Finlay 2004). Furthermore, their observation is largely laboratory-based and dependent on some technology, including microscopes, fixatives or stains. Fundamentally deprived of in-situ identifications and observations, almost determinant in a word of quick interactions (predation, infection, and reproduction), the spatiotemporal distribution of protists, has been largely disregarded, especially in the marine environment (Finlay et al. 2004). Maybe more out of ease than assurance, protists have been traditionally considered to be uniformly distributed in the ocean (Finlay & Clarke 1999, Foissner 2007). The great population size and apparent ubiquity of many protist taxa, was assumed to entail reduced differentiation among populations and low speciation rates (Fenchel & Finlay 2004, Livermore & Jones 2015). However, such explanations have been traditionally based on morphospecies, which might not reflect true diversity (Škaloud et al. 2019).

Backed by the same molecular tools that are revolutionizing the accurate identification of micro-eukaryotes, whilst sustaining a more stable cladistic classification; the "everything-everywhere" conception of protist biogeography is being challenged (Mann & Vanormelingen 2013, Leles et al. 2017). More recently, the "moderate-endemicity" model, which predicts a more limited dispersion of micro-eukaryote organisms based on adaptive, colonist, and geographical isolation (Foissner 2006, Foissner 2008, Bass & Boenigk 2011), is tractioning support (Grossman et al. 2016, Stürmer et al. 2018, Craig et al. 2019, Azovsky et al. 2020). The enormous cryptic diversity revealed among protist clades (**Introduction - Section 4**) is also transforming our understanding of protist communities in **space** and **time** (Aguillar et al. 2014, Leles et al. 2017, Caron & Hu 2019). This is, because the maturation of DNA processing and analysis techniques (NGS methods, computing resources, and bioinformatic tools) is consenting greater large-scale temporal analyses than ever (Massana et al. 2015, Boenigk et al. 2018, Oliverio et al. 2020). For instance, the already mentioned Tara Oceans expedition has shown significant variability in the micro-eukaryotic lineages constituting the planktonic community globally (Fig. 9). Moreover, in recent years, similar studies investigating other environments and including seasonal timescales are thriving (Trudnowska et al. 2020, Santoferrara et al. 2020, Schoenle et al. 2021).

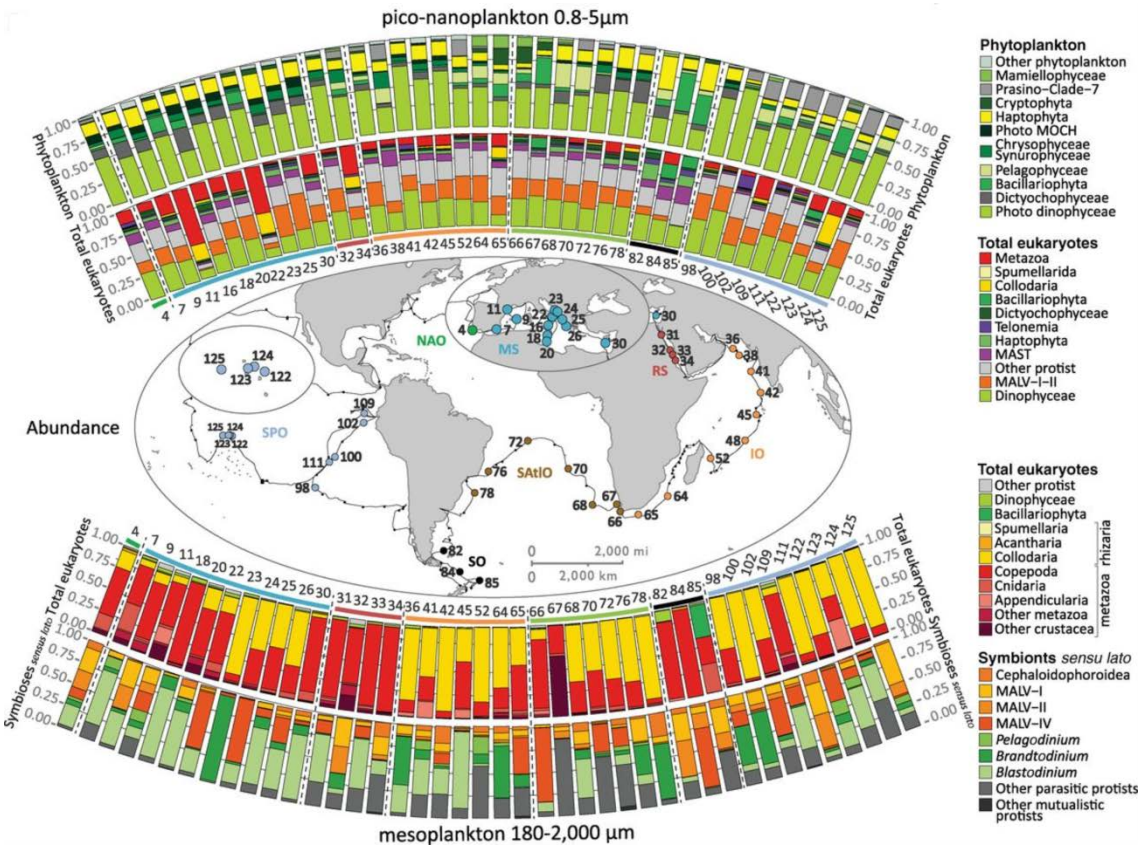

Fig. 9. Metabarcoding inference of trophic and symbiotic ecological diversity of photic-zone eukaryotic plankton. Relative abundance of major taxa across photic-zone eukaryotic plankton globally as shown from the Tara Oceans sampling stations (coloured dots in the map). **Above the map**, phytoplankton and all eukaryotes contituting piconanoplankton (0.8 – 5 µm). **Below the map** all eukaryotes and protistan symbionts (sensu lato) in mesoplankton. (from de Vargas et al. 2015).

The extent of the data generated by these metabarcoding experiments is so great that constricts methodologically and analytically the studies, which often have to narrow the spatiotemporal window or focus on the variability of a single or few major protists clades (Berdjeb et al. 2018, Renema 2018, Dabrowska et al. 2020). Apart from lineages sustaining photosynthetic activity and/or with potential to cause harmful algal blooms (Pawlowski et al. 2016, Tas & Lundholm 2017, Ilyash et al. 2018), the focus is often put on parasite-constituted clades (Cleary & Durbin 2016, Del Campo et al. 2019, Dumack et al. 2020, Anderson & Harvey 2020). However, a differential detection is one of the major setbacks of broad-targeted metabarcoding surveys (Santoferrara et al. 2020) that tend to underrepresent micro-eukaryote parasites (Bass et al. 2015, Williams et al. 2018), which have to be looked for with specific primers (**Introduction - Section 4**). Besides, parasites usually need to be adapted to both the host and the extracellular environment, reason why their spatiotemporal distribution patterns should be studied in conjunction with their hosts. For this reason, metabarcoding studies exploring the parasite community, especially pathogens, associated to a host; say, the **pathobiome** (Vayssier-Taussat et al. 2014, Bass et al. 2019) are emerging vigorously (Gómez-

Chiarri et al. 2015, Martínez-Porchas & Vargas-Albores 2017, Behringer et al. 2020, Holt et al. 2020).

Then again, the concept of pathobiome implicates a reduced or potentially reduced health status of the host (Bass et al. 2019), rendering visual techniques indispensable to demonstrate the pathogenic effect of a symbiont. Microcopy, isotopying, and other techniques such as In-Situ Hybridization, which allows merging visual and molecular approaches, are key to connect spatiotemporal variability detected through metabarcoding and actual disease dynamics in the environment, host, or population (Carnegie et al. 2003, Massana 2015b, Quince et al. 2017, Damm et al. 2020). As species with great commercial interest get prioritized (Holt et al. 2020), our comprehension of the spatial and temporal changes on the parasitic community associated to non-commercial invertebrates remains anecdotic (Hewson et al. 2013, Sweet & Bulling 2017). However, given their important role as vectors, intermediate hosts, and reservoirs, a much deeper comprehension on the changes of their pathobiome through time and space is key for a better prediction-power of pressure-factors, epidemics, or zoonoses.

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II. STATE OF THE ART,

HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

Most eukaryotes are single-celled organisms (protists), many of which belong to lineages that diverged early in the eukaryotic Domain. Microscopic, enormously diverse, and phenotypically convergent, their cladistic classification has been historically challenging, leaving behind an extensive record of paraphyletic and polyphyletic groups and associated taxonomy. Having no choice but to investigate ultrastructural, cell-biological, and ecological traits in a quickly-interacting microscopic world, protistology is particularly dependant on evolution-based systematics, as it enables to infer traits of concealed species from related taxa. In recent years, ever more refined molecular-based phylogenies are allowing to resolve long-standing questions on the evolution and speciation of protists. These, are bringing about fresh hypotheses on the derived characters and lifestyles shared by understudied species/lineages. Representing an accurate "record" of past speciation and diversification events, DNA (and RNA) molecules have turned indispensable to discriminate between morphologically similar taxa. Catalyzed by next generation sequencing (NGS) methods, ever larger multigene phylogenies are proving invaluable to trace the evolutionary history of protist lineages back to the last common ancestor of all eukaryotes (LECA). Concomitantly, molecular analyses based on shorter gene fragments (especially 18S rRNA), mainly recovered from environmental or organismal matrixes (eDNA), are opening a "Pandora´s box" of micro-eukaryotic diversity. Revelation of this "hidden" diversity is transforming our understanding of protist communities, particularly in the marine environment, where their role as autotrophs, heterotrophs, and myxotrophs, appears to be growing in importance by the day.

The concurrent surge in diversity and significance has been particularly pronounced among lineages of protists parasites, which adapted to life inside a host are more inaccessible and morphologically undistinguishable than their free-living counterparts. Very competitive as a lifestyle, current phylogeny-based analyses are showing parasitism to have evolved independently several times in virtually every single eukaryotic supergroup. In fact, it is increasingly regarded as one of the major, if not the most common, consumer strategy among living organisms. Moreover, the cumulative curve of cryptic species being discovered within most parasite-rich protist lineages is still rising steeply or only beginning to show a slow down. Noticeably, the discovery of this hidden diversity, including cryptic species, goes beyond the escalation in the number of species; it affects studies on cell-biology, lifecycles, or ecology.

Inexorably, this strength displayed by DNA sequencing and analysis methods, is opening up the gap between genetic diversity and our understanding of ultrastructure, pathology, host-

range, or transmission strategies of the putative parasites constituting it. The imbalance is particularly evident among parasites infecting invertebrate lineages, which excepting few taxa with commercial interest remain largely unscreened. Although the vast majority of animals in this planet are invertebrates, hosting possibly most of the existing parasite diversity, these connections have not been identified yet. However, many protist parasites infecting species of human concern (health or commercial interests) have heteroxenous lifecycles, often with invertebrates as vectors or reservoir hosts. Therefore, a better comprehension of the lifecycle of existing and cryptic protists parasite species, as they infect non-habitual but pivotal species in the ecosystem, can be determinant to understand when and where infection pressure on the host and/or the community might vary.

The progressive inclusion of parasites in ecological models is seeing far reaching variations in population and food-web dynamics. Correspondingly, parasite-host associations are increasingly regarded and investigated as a significant part of the community structure, rather than exclusively as a "nuisance". Regrettably, unlike for multicellular organisms, the spatial and temporal distribution of most unicellular eukaryote taxa remains a profound mystery. This is only but normal, as we can only track the spatial and temporal shifts of those organisms that we are able to observe and identify. The blurry and unstable classification and taxonomy of protists has rendered even more challenging the identification of these difficultly observable eukaryotes. Fundamentally deprived of in situ identifications and observations, almost determinant in a word of quick interactions (predation, infection, and reproduction), the spatiotemporal distribution of protists, has been largely disregarded, especially in the marine environment

Currently, one of the key goals for parasitology consists on investigating the community of pathogens associated to a host, the "pathobiome", through space and time. Then again, the concept of pathobiome implicates a reduced or potentially reduced health status of the host rendering visual techniques indispensable to demonstrate the pathogenic effect of a symbiont. Moreover, such techniques are key to connect spatiotemporal variability detected through metabarcoding and actual disease dynamics in the environment, host, or population. As species with greater health or commercial interest get prioritized, our comprehension of the spatial and temporal changes on the parasitic community associated to non-commercial invertebrates remains anecdotic. However, given their important roles as vectors, intermediate hosts, and reservoirs, a much deeper comprehension on their pathobiome and spatiotemporal variability is of paramount importance for a better prediction power of pressure factors, epidemics or zoonoses.

HYPOTHESIS

Common invertebrate species inhabiting the intertidal zone in temperate coastal ecosystems behave as cryptic reservoir hosts for a significant number of micro-eukaryotic (protist) parasites of concern for the marine environment and resources.

The progressive unearthing of these hidden parasite-host associations (by combined histopathological, ultrastructural, and molecular screenings) allows a better comprehension of the morphology, pathology, cell-biology, and lifecycle of pathogens, which in turn consents a closer monitoring of the factors and pressures driving epidemics and zoonoses in a spatiotemporal scale.

OBJECTIVES

The following objectives were established:

1- **To identify common and ecologically relevant invertebrate taxa in temperate coastal ecosystems** by means of an ecological assessment of the intertidal macrobenthos community in a selected type location in the South of the British Isles(**Chapter 1**)

2- **To determine which of these taxa might play a significant role as reservoirs of protist parasites** in the ecosystem by means of the histopathological and molecular analysis of host species collected during different seasons. (**Chapter 1**)

3- **To identify** and characterize by means of histopathology, ultrastructure, and/or molecular methods **the parasitic community associated to the main reservoirs** of protist symbionts, whilst developing a taxonomic guide describing target hosts(s), morphology, tissue tropism, pathology, and prevalence to guide future investigations.(**Chapter 1**)

4- **To determine spatiotemporal patterns in infection dynamics** through the characterisation of **natural variability of the parasite community associated to major reservoir hosts,** by conducting monthly histopathological screenings in the type location and seasonal samplings of comparable coastal locations in Southwest England. (**Chapter 1**)

5- To contribute to the morphological and molecular characterisation of the most relevant parasite taxa identified using light-microscopy, electron-microscopy, in-situ hybridization, phylogenetic, and phylogenomic analyses

6- To characterise the **lifecycle**, **cell-biology**, **tissue tropism**, **host range**, and potential **transmission strategies** of the most relevant parasite taxa identified, including comparative screening of macrobenthic species ecologically connected to major reservoir hosts.(**Chapter 2; Chapter 3**)

7- To define comprehensive cladistic classifications of major and understudied protists lineages, allowing the attribution of **hidden diversity** and its consideration as potential parasites of significance by constructing wide-ranging **phylogenetic** and **phylogenomic** trees using organismal and environmental DNA from coastal ecosystems throughout the British Isles, international sources, and global databases (**Chapter 2; Chapter 3**)

III. RESULTS AND DISCUSION

CHAPTER 1

Histopathological Screening of Common Invertebrate Taxa in the South Coast of the British Isles Reveals Amphipods as Key Reservoirs of Micro-Eukaryotic Parasites, a Spatial and Temporal Analysis

Abstract

The ecological characterization of Newton´s Cove, a narrow intertidal rocky beach in Weymouth (South British Isles), identified common and ecologically relevant species of the macrobenthic community in the area; these were later screened to examine their potential as reservoirs of significant micro-eukaryotic parasites. Four main taxa exerted dominance in the macrobenthic assemblages constituting the ecologically uneven habitat in the cove´s upper intertidal zone: *Echinogammarus* sp. (Amphipoda; Crustacea), *Capitella* sp. (Polychaeta; Annelida), *Procerodes* sp. (Turbellaria; Platyhelminthes) and ameirid harpacticoids (Copepoda; Crustacea). Trophically linked, all of them were greatly abundant (> 1000 individual m^{-2}). The histopathological analysis of these key invertebrate species revealed that amphipods hosted a considerable number of different micro-eukaryote parasites from lineages such as Ciliophora, Apicomplexa, Microsporidia, Endomyxa, Syndiniales, Oomycetes, or Filasterea. Including significant pathogens of commercially relevant invertebrates, infections caused protist parasites within these clades were monitored (monthly) in *Echinogammarus* sp. hosts, from April 2016 to September 2017. Concomitantly, two other amphipod genera present in this and other temperate coastal ecosystems (*Gammarus* and *Orchestia*) were screened, showing comparable parasitic communities but different parasite incidence and infection dynamics. Equivalent seasonal analyses of these three amphipod genera in other coastal locations in south-western England indicated a higher parasitic load in estuaries than in more exposed habitats, they also demonstrated the overlooked role of amphipods as reservoirs of known, hidden, and novel protist parasite diversity

Keywords: Macrobenthos, Protist, Symbiosis, Rhizarian, Alveolate, Stramenopiles, Opisthokonts, Microscopy, Network, Metabarcoding

1. Introduction:

The unremitting expansion of molecular biology has been attracting researchers from multiple disciplines to the study of protists, a world traditionally circumvented due to microscopic size, quick interactions, and ambiguous taxonomy (Cavalier-Smith 1995, Adl et al. 2007, Steele et al. 2011, Gao et al. 2019). Strongly dependant on identifying and characterizing some of the smallest and most cryptic eukaryotic organisms (Bass et al. 2015), the rampant progress in DNA sequencing techniques is making of protist parasitology one of the most deeply transforming fields (Cox 2009, Mahé et al. 2017). Genomic research is resolving long-standing questions about the evolution of micro-eukaryotes (Eme et al. 2017, Burki et al. 2020). Meanwhile, environmental DNA analysis is expanding the diversity of many parasitic micro-eukaryote lineages (Hartikainen et al. 2014, Pawlowski et al. 2016, Ward et al. 2018). Additionally, both approaches are increasingly used to investigate the spatial and temporal distribution of the parasitic community occurring in the environment and that associated with a host, the pathobiome (Vayssier-Taussat et al. 2014, Sweet & Bulling 2017, Bass et al. 2019). However, the concept of pathobiome, implicates a reduced (or potentially reduced) health status of the host, rendering microscopy and other visual techniques indispensable to demonstrate association between molecular detection of pathogenic lineages and actual disease (Pallen 2014, Bass & Del Campo 2020, Bateman et al. 2020).

Most eukaryotes including extant ones are singled celled organisms, many of which belong to lineages that diverged early in the eukaryotic Domain (Keeling & Burki 2019). Unlike their multicellular counterparts (animals, plants, or fungi) their microscopic size prevented for long a reliable taxonomic classification based on observable morphological traits (Scamardella 1999). While electron microscopy made possible the assignment of many species to cohesive phyletic groups (Sogin & Silberman 1998), only modern molecular techniques are allowing the disentanglement of the evolutionary relationships between them (Baldauf et al. 2000, Delsuc et al. 2005, Burki 2014, Keeling & Burki 2019). Although most lineages of micro-eukaryotes are still labelled as “protists” for practical purposes, Kingdom Protista Haeckel 1866 is known to be paraphyletic, with some lineages more closely related to multicellular animals, fungi, or plants than among them (Cavalier-Smith 2003, Adl et al. 2005). In fact, the archetypal classification of eukaryotes into Kingdoms has been substituted by an unranked tree, in which monophyletic clades are classified into “Supergroups” (Simpson & Roger 2004, Keeling et al. 2005, Adl et al. 2012). Animals, Fungi, and their unicellular relatives form lineage Opisthokonta (Karpov et al. 2014), which is evolutionarily related to amoeba (Amoebozoa) within supergroup Amorphea (Burki et al. 2020). In turn, primary photosynthetic eukaryotes (green plants, red algae, and glaucophytes) constitute supergroup Archaeplastida (Adl et al. 2005). The rest of single-

cell eukaryotic lineages are comprehended within Supergroups such as TSAR (Telonemids, Stramenopiles, Alveolates, Rhizarians), Haptista (Cavalier-Smith et al. 2015), Cryptista (Burki et al. 2016), CRuMS (Brown et al. 2018), Discoba (Hampl et al. 2009), or Metamonada (Cavalier-Smith 2003). Moreover, there are some "orphan" lineages with still unresolved phylogenies (Burki et al. 2020).

Resolving evolutionary relationships between protist lineages is determinant to understand their cell biology, ultrastructure, or ecology (Taylor 1999, Cavalier-Smith 2003, Torruella et al. 2015, Cadotte et al. 2017). Actually, the role played by micro-eukaryotes in aquatic ecosystems as autotrophs, heterotrophs, and mixotrophs is surging in significance as systematics progresses (Zubkov & Tarran 2008, Mitra et al. 2014). This mounting knowledge is clarifying their stance as a resource or a burden for human interests, especially as drivers of disease. Despite their damaging reputation, parasites play an irreplaceable role in the ecosystem moderating the population of highly abundant species (Tompkins et al. 2002) and consequently increasing biodiversity (Hatcher et al. 2012). However, this function is considered undesirable when parasitized hosts are humans or animals/plants with commercial relevance. Parasitism is a very competitive lifestyle (Combes 2001) that has evolved independently several times in essentially every single supergroup (Poulin 2011). There are important parasitic lineages among animals, like Myxozoa, Nematoda, or Trematoda (Poulin 2006, Blaxter & Koutsovoulos 2015, Holzer et al. 2018), and all major fungal clades (Takamatsu 2004). Even among photosynthetic archaeplastids parasitic lineages can be found (Brooks 2004, Procházková et al. 2015). However, the greatest diversity and number of eukaryotes with a parasitic lifestyle belong to unicellular lineages (Skovgaard 2014).

Among opisthokonts, unicellular clades such as Ichthyosporea, Microsporidia, Aphelidea, Chytrids, or Cryptomycetes are important pathogens of animals, plants, fungi, and other protists (Mendoza et al. 2002, Kagami et al. 2007, Karpov et al. 2014, Frenken et al. 2017, Bass et al. 2018). Amoebozoan lineages Archamoebae and Mycetozoa include the causative agents of some of the most significant protist infections in fish and shellfish aquaculture (Rodger & McArdle 1996, Dyková et al. 2007, Small & Pagenkopp 2011, Sühnel et al. 2014). Some concerning human pathogens like *Trypanosoma* sp. and *Leishmania* sp. can be found in lineages Discoba and Metamonada, which also include significant protist parasites for marine vertebrates and invertebrates (Monis et al. 1999, Cepicka et al. 2006). The TSAR supergroup is probably the largest, at least in number of protists species (Adl et al. 2007), and it includes numerous and very significant parasitic lineages of farmed and wild aquatic animals, such as Endomyxa, Apicomplexa, Syndiniales, Perkinsozoa, Ciliophora, or Oomycetes (Sierra et al. 2016, Guillou et al. 2008, Morrison 2009, Beakes et al. 2012).

A considerable number of these and other micro-eukaryotic parasitic lineages have been described or documented from coastal ecosystems in the British Isles, often associated to pathology and mortality events in marine animal hosts (Stentiford et al. 2002, Paley et al. 2012, Longshaw et al. 2013, Rodger 2014, Stentiford et al. 2019). Moreover, environmental DNA studies have shown that a significant number of these parasitic clades are much more diverse and abundant than previously estimated (Beck et al. 2009, Hartikainen et al. 2014, Jephcott et al. 2016, Ward et al. 2016, Bass et al. 2019). However, while a rampant progression in sequencing techniques and associated bioinformatic tools is redefining the phylogeny, diversity, and community composition of parasitic protists (Jeunen et al. 2019), our understanding of their ultrastructure, histopathology, behaviour, or cell biology is lagging behind (Caron et al. 2017, Tashyreva et al. 2018). Naturally, these research lines are prioritizing the study of micro-eukaryotes affecting humans and farmed species, including fish and shellfish aquaculture (Jenkins et al. 2015, Lafferty et al. 2015, Koutsoumanis et al. 2018, Behringer et al. 2020). However, many of these pathogens (and clades) have complex life cycles often constituted by a myriad of reservoirs, vectors, and intermediate hosts that remain largely unidentified, especially in the marine environment (Small & Pagenkopp 2011, Morand 2018).

Possibly, one of the most significant and less studied reservoirs of protists parasites in aquatic ecosystems is indeed the macrobenthos, a term that embodies the invertebrate community (> 1mm) living within the superficial layer of the sediment in marine and freshwater environments (Martin et al. 2005, Kelly et al. 2009). Excluding some significant shellfish species (Vezzulli et al. 2018, Davies et al. 2019, Behringer et al. 2020), the pathobiome associated to most invertebrate species inhabiting coastal ecosystems remains uncharacterized. Accordingly, our understanding of the spatial and temporal variation of the disease-causing pathogens sustained within these reservoirs is anecdotic (Cohen & Bishop 2015, Berdjeb et al. 2018, Anderson & Harvey 2020). However, the macrobenthos represents the most widely used component in environmental impact studies (Pearson & Rosenberg, 1978, Pocklington & Wells, 1992, Mucha et al. 2003). The reason is that this part of the infauna is relatively non-mobile, and therefore responds to local effects allowing the investigation of both short- and long-term environmental events at a particular place (Holme & McIntyre, 1971). Thus, exploring the pathogen community associated to benthic marine invertebrates is desirable, not only to understand better local disease dynamics and infection outbreaks, but to consider additional biotic variability in toxicological and environmental analyses (Grassman 2002, Marcogliese & Pietrock 2011, Sures et al. 2017).

This study aimed to identify common invertebrates of the intertidal zone that might constitute potential reservoirs of significant protist parasites in the southwest English coast. To begin with, an ecological analysis of a selected type-location (Newton's Cove, Weymouth, UK) was conducted to

characterize the macrobenthic community inhabiting the intertidal zone in this area. Following, the most abundant invertebrates observed (*Echinogammarus* sp., *Capitella* sp. *Procerodes* sp., and harpacticoid copepods) were histopathologically screened for parasites, paying especial attention to micro-eukaryotes. This pilot screening indicated that the *Echinogammarus* sp. population was particularly affected by a number of unidentified microcell lineages. Consequently, a two-year longitudinal study of this and other common amphipod species present in Newton's Cove was carried out to define the main pathogens and diseases affecting these key crustaceans in aquatic environments through time. Histology, ultrastructure, and phylogeny were analysed to identify species and establish potential connections with other elements of the infauna present in the coastal area. Temporal variability in the prevalence of each of the protist infections was investigated and compared among host species, looking for possible explanations and connections with biotic/abiotic factors. To conclude, three more sampling locations in the southwest coast of England (Tamar, Dart, and Camel estuaries) were compared during different seasons in order to observe wether disease patterns observed in amphipods from Newton's Cove were extrapolatable to a wider area of the English Channel.

2. Materials and methods:

2.1 Ecological analysis of Newton's Cove

2.1.1 Study area

Newton's Cove is a narrow intertidal rocky beach located between Portland's harbour and Weymouth's beach, in southwest England (Fig. 1). Its proximity to the Centre for Environment Fisheries and Aquaculture Science (Cefas) laboratory, ample tidal range (~2.48 m), and heterogeneous vertical profile (including rocky areas, with sandy and muddy substrates and regularly scattered ponds) made it a suitable location for regular (monthly) samplings.

An aerial image obtained by Cefas´ RPA (remote piloted aircraft) was used in combination with an *in-situ* analysis of the cove, to define a representative and heterogeneous section of this part of the coast (Fig. 1B). The selected area was 60 m long and 40 m wide and comprised the upper and lower limits of the intertidal range (Fig. 1C). The 2400 m^2 partition was further divided into 4 m^2 parcels. The exposure time (air) of each of the parcels, was measured taking as reference the upper limit of the high tide. A laser distance-meter was used to identify the exact position of each quadrant using the walls delimiting the beach as a reference. The"tilt" function of the device was used in combination with a scaled rod, to estimate the relative elevation of each quadrant in relation to the tide. These measurements were used to produce a 3D map of the selected area in Newton's Cove in

Surfer 7.0 (Golden Software LLC). This altimetric-type map of emersion was combined to a visual assessment of the substrate and algal coverage to pre-define five different habitats in the beach (Fig. 1D), in order to conduct a stratified random sampling.

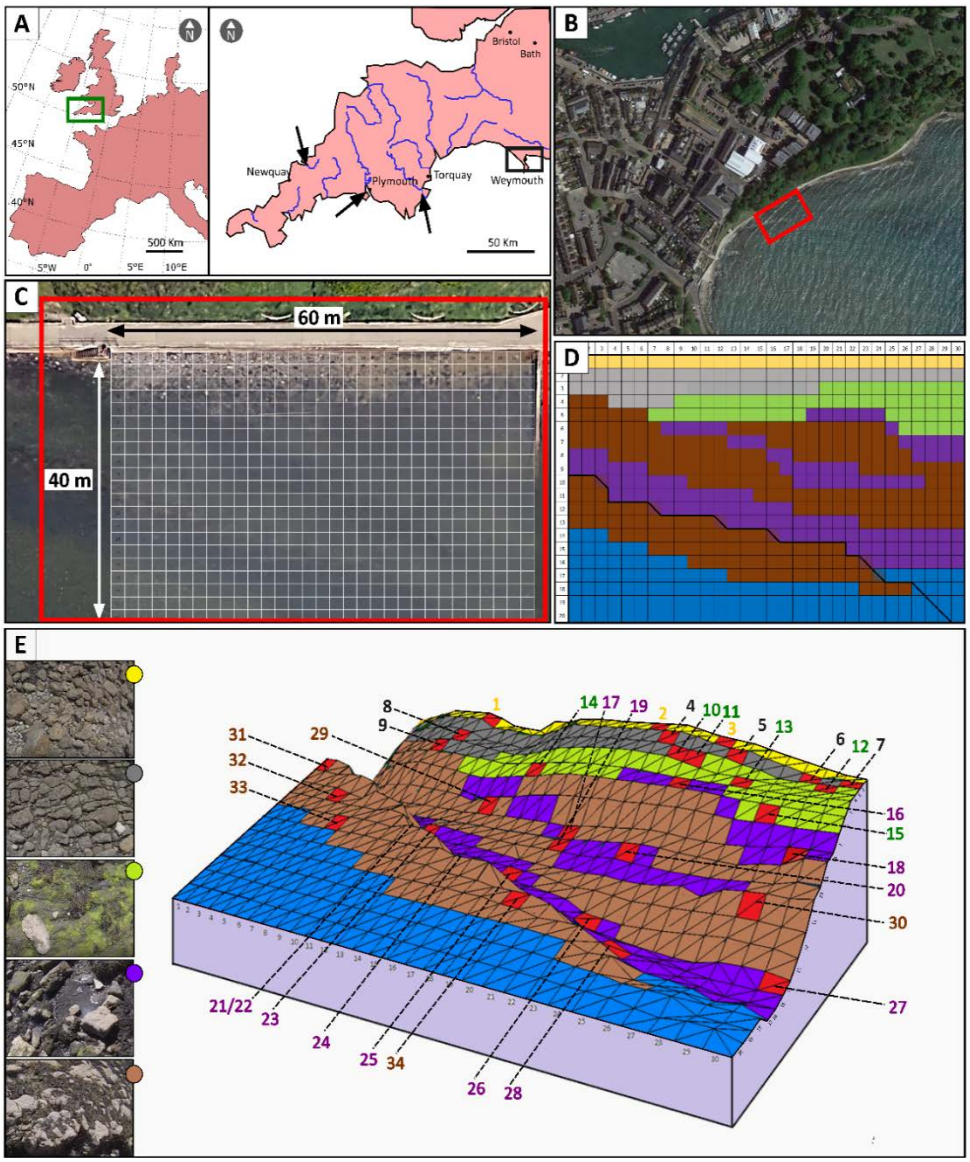

Figure 1: Sample collection sites and morphometric description of Newton's Cove (Weymouth), a model spot partitioned in microhabitats. **A)** Sample collection siteswere located in Southwest England (arrows). North of Newquay lies the Camel estuary;Tamar estuary passes through Plymouth;south of Torquay, in Dittisham, the Dart estuary. The majority of samples for this study were collected in a sea-exposed rocky beach in Weymouth called "Newton's Cove"(black square), selected as model location that was morphometrically and ecologically analysed. **B)** The area selectedin Newton's Cove (red square) covers all the intertidal range and is characterized by a heterogeneous substrate. **C)** A 60 m long and 40 m wide stretch was selected and divided into 4 m^2 quadrants. **D)** Each of the (2 m x 2 m) quadrants were visually assessed and classified intoone of five different microhabitats: boulders over a gravel/naked-rock substrate(yellow area);naked rock partially covered by gravel and/or small stones (grey area); naked rock partially covered by algae (green area); big boulders and shallow ponds in the lower intertidal (purple area); exposed zone in the lower intertidal covered by algae (fucoids) attached to the naked rock. **E)** Randomly selected quadrants (coloured in red) sampled and an image showing the "type" substrate in each microhabitat.

In the upper part of the intertidal, a narrow belt (60 m long, 2 m wide), the so-called "Yellow area", was constituted by pilled boulders lying over a substrate formed by smaller stones and decomposing wrack. Immediately after, still in the upper part of the intertidal was the "Grey area", with fewer and smaller boulders over a predominantly muddy sediment. Certain quadrants forming this "grey area" belt contained barely any sediment, with smaller stones laying directly over the naked rock. The habitat branded as the "Green area" marked the lower limit of the upper intertidal; sparsely scattered boulders laid over the naked rock, which was partially covered by the first algal assemblages, predominantly belonging to genera *Blindingia*, *Ectocarpus*,*Ulothrix* and *Ulva*. The "Purple area" was defined by the alternation of emerged areas and shallow ponds. *Fucus vesiculosus* dominated this zone, although *Chondrus* sp. and *Corallina* sp. were also significant. Finally, the "Brown area" corresponded to quadrants falling within emerged ridges/elevations in the middle and lower intertidal zones. In this area, big boulders were sparsely distributed over the bear rock, which was almost completely covered by *Ascophyllum nudosum* and *Fucus serratus.* With *Laminaria* sp. in the lowest-levels.

2.1.2 Sample collection and identification:

The number of quadrants sampled for every zone was proportional to the relative size of each niche. The 4 m^2 quadrants were randomly selected (RStudio v1.1.383), and further divided into 64 smaller squares (side = 0.25 m). A second random number was used to choose which of the smaller squares was sampled in each quadrant. All organisms enclosed within the metallic grid (0.25 x 0.25 m) except vertebrates (fish), were collected in independent containers and taken to the laboratory. Using a stereo-microscope, all organisms were identified, counted, and separated into smaller pots containing 100% alcohol, with the exception of harpacticoid copepods, which were not counted when n > 200 individuals. Consequently, they were excluded from the subsequent ecological analysis. The following guides and websites were used to classify each of the sampled organisms to the utmost taxonomic level allowed by our expertise: Handbook of The Marine Fauna of North West Europe (Hayward & Ryland 2017), Marine Species Identification Portal (www.species-identification.org), MarLIN (www.marlin.ac.uk), MarBEF (www.marbef.org) and WORMS-World Register of Marine-species (www.marinespecies.org). More specific identification keys were used for nematodes (Platt and Warwick 1988), molluscs (Thompson 1988, Graham 1988), annelids (Brinkhurst 1963, Radashevsky 2012), arthropods (McCafferty 1983, Palerud & Vader 1991, Bartsch 2006, Vives et al. 2007, Wells 2007), nemerteans (Gibson et al. 1982) and echinoderms (Picton 1993).

2.1.3 Data management and ecological analysis

The RStudio-implemented Vegan package (Oksanen et al. 2007) in conjunction with the MASS library was used to calculate diversity indexes and ordinate the data of species and abundances for each quadrant. Species accumulation curves were estimated for each of the five habitats separately and jointly, using Kindt's exact method under the "specaccum" function. The ranked abundance distribution analyses and graphs were generated using "radfit" function. Differences between the 34 sampled quadrants in species occurrence and abundance (Bray-Curtis dissimilarity) were calculated using the MDS algorithm (isoMDS function); and presented in a non-metric multidimensional scaling analysis (NMDS) built using the "ordiplot" function. A principal component analysis (PCA) of the sites, using species (and their abundance) as variables was built using "rda" function in Vegan. The occurrence probability (single extraction) for every species identified in Newton's Cove was calculated independently for each habitat and represented as a heatmap, using the "heatmap" function in RStudio.

2.2 Screening for protist parasites infecting key invertebrate species in Newton's Cove

2.2.1 Sampling campaigns

Invertebrate species observed to be highly abundant in Newton's Cove (*Echinogammarus* sp., *Capitella* sp., *Procerodes* sp., and harpacticoid copepods) were collected at different times of the year for histological and molecular screening (Table 1).

2.2.2 Histological procedures

The head and few immediate segments of *Capitella* sp. polychaetes were transversally sectioned and preserved in 2.5% glutaraldehyde for electron-microscopical analysis. So were the head and first 2 or 3 pereonic (thoracic) segments of the amphipod *Echinogammarus* sp. The rest of the body (about three quarters) of the polychaete and the amphipod was immediately fixed in Davidson's seawater fixative (Hopwood 1969) for 24 h, and then transferred to 70% ethanol for histological analysis to the light microscope. The procedure was identical for the turbellarian *Procerodes* sp. except for the sectioning, which was conducted in a sagittal plane, with half of the body being fixed in Davidson's and the other half in glutaraldehyde. Copepods were imbedded (whole body) in agar (Feist & Bucke 1983) after fixation in Davidson's fixative, to facilitate their concentration and/or provide a particular orientation.

Samples were then processed from ethanol to wax in a vacuum infiltration processor using established laboratory protocols (Stentiford et al. 2013). Tissue sections were cut at a thickness of 2.5-3 µm on a Finnese® microtome, left to dry for 24 h, mounted on VWR™ microscope slides, and stained with H&E (Bancroft & Cook 1994). Cover-slipped sections were examined for general histopathology by light microscopy (Nikon Eclipse E800). Digital images and measurements were obtained using the Lucia™ Screen Measurement software system (Nikon, UK).

For electron-microscopy, glutaraldehyde-fixed samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in epoxy resin 812 (Agar Scientific pre-Mix Kit 812, Agar scientific, UK) and polymerised overnight at 60 °C. Semi-thin (1 µm) sections were stained with 1% Toluidine Blue and analysed by light microscopy to identify target areas containing sufficient parasites. Ultrathin sections (70-90 nm) were framed on uncoated copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1400 transmission electron-microscope and digital images captured using a GATAN Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.2.3 DNA extraction and polymerase chain reaction

Independent batches of *Echinogammarus* sp., *Procerodes* sp., *Capitella sp.*, and copepods (Table 1) were isolated and preserved in 100% molecular grade ethanol (Fisher BioReagents™). Tissues were disrupted and digested overnight (12 hours) using Fast Prep® Lysing Matrix tubes containing 0.2 mg (6 U) Proteinase K (Sigma-Aldrich®) diluted 1/40 in Lifton's Buffer (100 mM EDTA, 25 mM Tris-HCl, 1 % (v/v) SDS, pH 7.6). Next, a 1/10 (v/v) of 5 M potassium acetate was added to each of the tubes containing digested sample, Proteinase K, and Lifton's buffer. The solution was mixed and incubated on ice for 1 hour. From here DNA was extracted using the phenol-chloroform method described in (Sambrook et al. 1989). The resulting pellet was diluted in 50 µl of molecular grade water and DNA concentration quantified using NanoDrop™ (Thermo Fisher Scientific). Batches of invertebrates contained within tubes were examined for the presence of the rRNA of certain protists parasites identified by histology using specific primers and PCR conditions established in (Table 2). A total reaction volume of 50 µl included 30.75 µl molecular water, 10 µL GoTaq® Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide, 40 pM of each primer, 1.25 U GoTaq® Polymerase (Promega) and 200 ng of extracted DNA. Finally, PCR products were run on a gel electrophoresis (1% Agar) and images captured using a Xr^+ Gel-Documentation system™ (Bio-Rad Laboratories, Inc.)

Table 1: Sampling information for the invertebrates collected in Newton's Cove (Weymouth) for histopathological and PCR screening of parasites.

Sampling location	Date	Organism	Histology (Davidson's)	Molecular techniques (ethanol)
Newton's Cove, Weymouth, UK. (Upper intertidal) Coordinates: 50° 36' 17'' N 02° 27' 03'' W	21-May-19	*Capitella* sp.	n = 20	n = 45 (3 tubes, 15 ind. each)
		Procerodes sp.	n = 20	n = 60 (2 tubes, 30 ind. each)
		Echinogammarus sp.	n = 20	n = 100 (4 tubes, 2 x 20 big; 2 x 30 small)
		Copepoda	n = 0	n = 300 (2 tubes, 150 ind. each)
	10-Jul-19	*Capitella* sp.	n = 20	n = 90 (3 tubes, 30 ind. each)
		Procerodes sp.	n = 20	n = 90 (3 tubes, 30 ind. each)
		Echinogammarus sp.	n = 20	n = 100 (4 tubes, 2 x 20 big; 2 x 30 small)
		Copepoda	n = 20	n = 400 (2 tubes, 200 ind. each)
	05-Ago-19	*Capitella* sp.	n = 20	n = 120 (4 tubes, 30 ind. each)
		Procerodes sp.	n = 20	n = 120 (4 tubes, 30 ind. each)
		Echinogammarus sp.	n = 20	n = 100 (4 tubes, 2 x 20 big; 2 x 30 small)
		Copepoda	n = 20	n = 100 (1 tube)
	28-Sep-19	*Capitella* sp.	n = 20	n = 120 (4 tubes, 30 ind. each)
		Procerodes sp.	n = 20	n = 120 (4 tubes, 30 ind. each)
		Echinogammarus sp.	n = 20	n = 100 (4 tubes, 2 x 20 big; 2 x 30 small)
		Copepoda	n = 20	n = 500 (2 tubes, 250 ind. each)
	15-Nov-19	*Capitella* sp.	n = 20	n = 90 (3 tubes, 30 ind. each)
		Procerodes sp.	n = 20	n = 90 (3 tubes, 30 ind. each)
		Echinogammarus sp.	n = 20	n = 100 (4 tubes, 2 x 20 big; 2 x 30 small)
		Copepoda	n = 20	n = 200 (2 tubes, 100 ind. each)

Table 2: Primers used for PCR amplification of the DNA of parasitic protists lineages infecting invertebrates in Newton's Cove (Weymouth).

Parasite	PCR type	Primer	Gene	Sequence	PCR conditions	Band length	Reference
Haplosporidia	nPCR (1st round)	C5fHapI	(V6 - V9) 18S rRNA	5'-GTA GTC CCA RCY ATA AAC BAT GTC-3'	95 °C (3 mins) + 35 cycles {95 °C (45 s), 65 °C (60 s), 72 °C (60 s)} + 72 (10 mins	~ 750 bp	Hartikainen et al. 2014
		Sb1n		5'-GCA TCC ACT TGG ACG YCT HCC TAG-3'			
	nPCR (2nd round)	V5fHapI		5'-GGA CTC RGG GGG AAG TAT GCT-3'		~ 650 bp	
		Sb2nHap		5'-CTC CTT CYT BTT CAG CAT TGT TCC-3'			
Paramyxea	nPCR (1st round)	Para1 + fN	(V6 - V8) 18S rRNA	5'-GCG AGG GGT AAA ATC TGA T -3'	94 °C (3 mins) + 42 cycles {94 °C (45 s), 67 °C (45 s), 72 °C (30 s)} + 72 (5 mins)	~ 750 bp	Ward et al. 2016
		ParaGenrDB		5'- GTG TAC AAA GGA CAG GGA CT-3'			
	nPCR (2nd round)	Para3 + fN		5'-GGC TTC TGG GAG ATT ACG G -3'	94 °C (3 mins) + 42 cycles {94 °C (45 s), 62 °C (45 s), 72 °C (30 s)} + 72 (5 mins)	~ 480 bp	
		Para2 + rN		5'- TCG ATC CCR ACT GRG CC -3'			
Microsporidia	standard PCR	V1F	18S + ITS1 rRNA	5'-CAC CAG GTT GAT TCT GCC TGA CG-3'	98 °C (5 mins) + 40 cycles {98 °C (30 s), 63 °C (30 s), 72 °C (30 s)} + 72 (10 mins	~ 1220 bp	Vossbrinck et al. 1987 McClymont et al. 2005
		1342r		5'- ACG GGC GGT GTG TAC AAA GAA CAG-3'			
Parasitic Filasterea	standard PCR	S47-152F	(V1 - V4) 18S rRNA	5'-AGC TAA TAC ATG CTG CAA AGC GG-3'	95 °C (5 mins) + 35 cycles {95 °C (30 s), 67 °C (30 s), 72 °C (90 s)} + 72 (10 mins	~ 500 bp	This study
		S47-617R		5'-CGC TTT CAC GCG ACC ATC ACA AC-3'			
Syndiniales (Dinoflagellates)	nPCR (1st round)	ITS1F	ITS-1 + 5.8S + ITS-2 rRNA	5'-AAC CTG CGG AAG GAT CAT TC-3'	94 °C (5 mins) + 35 cycles {94 °C (30 s), 56 °C (30 s), 72 °C (45 s)} + 72 (5 mins)	~ 500 bp	Small, H. PhD VIMS (USA) Pers. Comm.
		ITS1&2R		5'-CCG AGC CGA GGC ATT CAT CGC T-3'			
	nPCR (2nd round)	ITS1F		5'-AAC CTG CGG AAG GAT CAT TC-3'		~ 380 bp	
		ITS1R		5'-TAG CCT TGC CTG ACT CAT G-3'			

2.3 Spatial and temporal variation of protists parasites infecting amphipods in southwest UK

2.3.1 Sampling locations and campaigns

Amphipods were regularly sampled in Newton's Cove from April 2016 to August 2017, and occasionally in the Tamar, Dart, and Camel estuaries (Fig. 1A). Highly abundant in the upper part of the intertidal *Echinogammarus* sp. was sampled more frequently and in higher numbers than genera *Gammarus* and *Orchestia* (Table 3). Amphipods belonging to genera *Melita* and *Jassa* were also sporadically sampled but were not included in the posterior comparative analysis.

Table 3: Amphipods collected for full histopathological screening (light microscopy, electron microscopy, and molecular analysis. Number of individuals belonging to different genera are linked to location and sampling date.

Sampling location	Location coordinates	Date	Number of amphipods sampled		
			Echinogammarus sp.	*Gammarus* sp.	*Orchestia* sp.
Dart Estuary	50° 23' 21'' N 03° 35' 36'' W	19-Sep-16	64	10	31
		26-Apr-17	40	6	21
		13-Aug-18	41	0	0
		25-Feb-19	34	0	0
Tamar estuary	50° 23' 25'' N 04° 13' 51'' W	20-Sep-16	84	0	28
		27-Apr-17	41	27	37
		14-Aug-18	48	0	34
		08-Nov-18	35	7	27
		26-Feb-19	44	0	0
Camel Estuary	50° 32' 17'' N 04° 56' 05'' W	28-Apr-17	30	29	37
Newton's Cove (Weymouth)	50° 36' 17'' N 02° 27' 03'' W	20-Apr-16	50	0	0
		08-Jun-16	30	0	0
		21-Jul-16	32	0	0
		13-Sep-16	38	30	32
		28-Oct-16	30	20	0
		25-Nov-16	40	0	0
		14-Dic-16	63	42	0
		17-Jan-17	40	30	0
		16-Feb-17	50	23	0
		16-Mar-17	54	0	6
		11-Apr-17	38	15	0
		04-May-17	51	0	0
		18-May-17	31	0	0
		15-Jun-17	12	10	25
		21-Jul-17	40	10	23
		16-Mar-18	55	12	10
		16-Apr-18	45	8	4
		11-May-18	31	0	0
		13-Jun-18	55	0	0

2.3.2 Histological procedures

The head and first two pereonic segments of amphipods *Echinogammarus* sp., *Orchestia* sp. and *Gammarus* sp. were transversally sectioned and preserved in (molecular grade) ethanol for molecular analysis. The immediate pereonic segment was fixed and maintained in 2.5% glutaraldehyde for electron-microscopical analysis. The rest of the body was fixed in Davidson's seawater fixative for 24

hours, and then transferred to 70% ethanol for histological analysis. From here, processing for light- and electron-microscopical analyses was conducted as described in section 2.2.2.

2.3.3 Molecular procedures

Tissues (head and immediate segments) from amphipods observed to be infected by protists parasites (light microscopical analysis) were digested, and their DNA extracted as described in section (2.2.3). Partial rRNA gene sequences from the different parasites were amplified using primers and conditions established in Table 2. Amplicons were run on a gel electrophoresis and examined as in section 2.2.3, but bands with lengths in the range estimated for each parasite (Table 2) were dissected and cleaned using 20 % polyethylene glycol 8000 (Sigma-Aldrich®) followed by an ethanol precipitation. A total volume of 15 µl was mixed with 2 µl of the forward primers, before being single-read Sanger sequenced (Eurofins®Genomics).

The DNA of five amphipods (*Echinogammarus* sp. and *Gammarus* sp.) was shotgun-sequenced (Hiseq or Miseq Illumina platforms). FASTQC (Andrews 2010) was used to assess the quality, length, GC content, and number of paired and unpaired reads. Forward and reverse reads were trimmed for adaptor sequences, contamination, and low-quality reads, using Trimmomatic v0.39 (Bolger et al. 2014), and the following parameters (Leading: 3, Trailing: 3 Minlen: 36). The assembly of the resulting trimmed reads was carried out by MEGAHIT (Li et al. 2015) using default parameters. CheckM (Parks et al. 2015) and BUSCO (Simão et al. 2015) were used to identify 18S genes in the assembly and their taxonomic position. A fast alignment of contigs (assembled fragments) containing eukaryotic 18S genes was conducted using Diamond v0.9.29.130 (Buchfink et al. 2015). Specific contigs of interest were BlastN searched (Zhang et al. 2000) for identity and coverage computation.

2.3.4 Phylogenetic analysis

The PCR amplified (18S, 5.8S, ITS1, or ITS2) rRNA genes of microsporidian, paramyxid, and syndinian parasites infecting *Echinogammarus* sp., *Gammarus* sp. and *Orchestia* sp., were independently BlastN-searched (Zhang et al. 2000) against the GenBank nucleotide (nt) database. Reference sequences showing highest similarity to each of the parasites, as well as related taxa were downloaded and independently aligned using MAFFT (Katoh et al. 2007) and the L-ins-I algorithm. In the case of the microsporidian and the paramyxid, alignments only included the 18S rRNA gene. For the syndinian parasite, the alignment consisted of a concatenation (18S, ITS1, 5.8S, ITS2) of genes. Alignments were trimmed with TrimAL (Capella-Gutiérrez et al. 2009) using the “automated-1” option. The GTR + F + G model was used to generate a Maximum Likelihood (ML) tree in IQ-TREE v.1.6.10 (Nguyen et al. 2015). A second maximum likelihood phylogenetic tree was constructed using RAxML v.8.2.12 (Stamatakis

2014). Support values calculated using 1,000 bootstrap replicates were mapped into a tree with the highest likelihood value (evaluated under GTRGAMMA model). A Bayesian inference consensus tree was built using MrBayes v.3.2 (Ronquist et al. 2012) under default parameters except for the following: the number of substitution types was mixed, the model for among-site rate variation, Invgamma; the use of covarion like model, activated. The MCMC parameters changed were: 5 million generations; sampling frequency set to every 1,000 generations; starting tree set to random; and all compatible groups consensus tree. A final tree figure was created using FigTree v.1.4.3 (Rambaut 2017) based on the Bayesian topology.

2.3.5 Data management and graphical analysis

The apparent prevalence of the parasitic protist identified by histology, electron-microscopy or molecular techniques was computed for each species, month, and location. Additionally, identifiable non-protist (metazoan, fungi) parasites/symbionts were also included in the analysis to examine possible co-occurrence, and their potential role as vectors. When detected, the incidence of bacterial and viral infections was also documented. The sex of individual amphipod hosts was examined during dissection and histologically. Intersex individuals and female bearing larvae were determined histologically. Temporal variation in the parasitic community infecting amphipods in Newton's Cove was matched to oscillation in the seawater temperature (Cefas deployed buoy system: www.cefas.co.uk). A PCA was generated using the "rda" function in Vegan package to observe the influence of each parasite in the variability observed between host species and sampling dates in Newton's Cove. Co-occurrence of parasites in each amphipod taxa was examined graphically using the "igraph" package implemented in RStudio (Csardi & Nepusz 2006) and the Fruchterman-Reingold force-directed algorithm (500 iterations, 22.737 starting temperature). Pairs of parasites emerging as co-occurring in the network were statistically examined using Pearson's chi-squared test implemented in RStudio. Accumulated prevalence (the sum of prevalences for every parasitic lineage (protists and non-protists) was independently computed for each host species, sampling location (Newton's Cove, Dart, Tamar and Camel estuaries), and season.

2.3.6 Statistical analysis

Statistical comparison of the accumulated incidence of parasites for different hosts, locations, and seasons, was conducted using RStudio. Homogeneity of variance (Levene's Test) and normality (Shapiro-Wilk Test) were tested before statistical analysis. For non-normal distributions, the Kruskal-Wallis Test was used as an alternative to the one-way Anova implemented for normally distributed datasets. The Tukey Test was used to identify which pair(s) of groups made up for the significant

differences observed in the one-way Anova. Alternatively, the Pairwise Wilcox Test was used to identify which groups made up for significant differences revealed by Kruskal-Wallis Test in non-normally distributed data. The level of significance considered for all analyses was $\alpha = 0.05$.

3. Results:

3.1 General ecology of Newton's Cove

In total, 34 parcels of 0.0625 m^2 (0.25 m x 0.25 m) were analysed from the 2400 m^2 area of interest defined in Newton's cove (Fig. 1). Altogether, 65 different entities (62 species) were identified in-situ or in a posterior lab-based examination of each quadrant. The species-accumulation curves produced for each of the five biomes defined in the location ("Yellow", "Grey", "Green", "Brown", and "Purple" areas) were still increasing (Fig. 2A), indicating a number of unseen species, especially in the lower part of the intertidal (habitats designated as "Brown" and "Purple" areas). A similar outcome was achieved when the cumulative number of species by sampled area (number of sites), was analysed for the five habitats combined (Fig. 2B). Despite the reduced slope, the curve still showed that several species remained undiscovered. The cumulative number of species identified in half of the quadrants examined (n = 17) was 48 (Fig. 2B), a 77 % of the total.

Ordination of identified species in rank-abundance curves (Fig. 2C) showed matching arches in the uppermost ecosystems, the so-called "Yellow" and "Grey" areas. In this upper zone of the intertidal few species accounted for most of the abundance observed. The curve's tilt commenced to decrease in the "Green" area, where the first algal assemblages appeared over the rocky substrate in Newton's cove. The best fitting model changed from "pre-emption", observed in the upper rocky part of the intertidal to "Mandelbrot". This distribution model fitted best the lower niches of the intertidal as well, which are characterized by a significant increase in the species richness observed but a lower abundance. The species rank-abundance curve generated for all niches combined (Fig.2D), showed that four organisms (*Echinogammarus* sp., *Capitella* sp. *Procerodes* sp. and harpacticoid copepods (Family Ameiridae) accounted for a significant quota of abundance observed in Newton's Cove. All of them surpassing the threshold of 1000 individuals per total area sampled (2.125 m^2).

The non-metric multidimensional scaling comparison of the species observed in each of the 34 quadrants analysed (Fig. 2E) showed that the predefined "Yellow", "Grey", and "Green" areas, were not clearly distinguishable from each other, at least based on a two-dimensional representation. However, there was a very marked dissimilarity between the communities observed in these three zones of the upper intertidal and those constituting the lower intertidal. Furthermore, the second axis (NMDS 2) appeared to separate communities from "brown" and "purple" areas. Both of them had very similar rank-abundance curves, but the species composition was different.

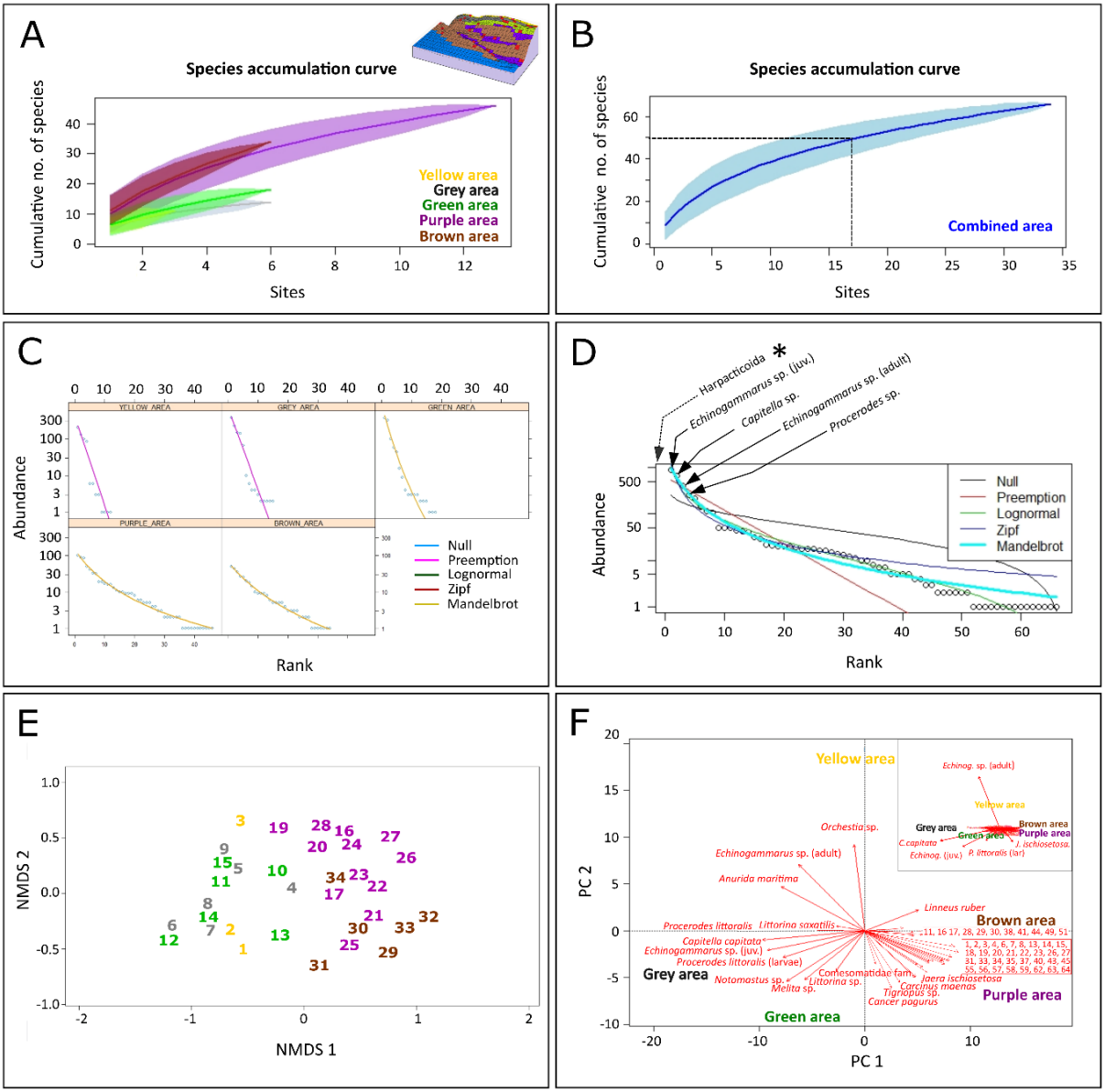

Figure 2: Multivariate ecological analysis of the invertebrate community inhabiting Newton's Cove. **A)** Independentspecies accumulation curves for each of the five microhabitats defined in (Fig. 1). The cumulative number of species identified (Y axis) is set against the number of quadrants sampled (X axis). **B)** Species accumulation curve for all five microhabitats combined. The cumulative number of species (Y axis) is opposed to the total quadrants sampled (X axis). **C)** Rank-abundance diagramsfor species collected in the different microhabitats and best fitting model for each distribution. **D)** Rank-abundance diagram displaying total number of specimens collected for each species (Y axis) organized by rank (from more to less abundant). The five most abundant organisms observed in Newton's Cove are indicated with arrows. Harpacticoid copepods (*) are shown by a discontinuous arrow as their exact number (>200 in several quadrants) was excluded from the analysis). **E)** Non-metric multidimensional scaling (NMDS) of each of the 34 quadrants sampled (Fig. 1) based on Bray-Curtis dissimilarity of the species found and their abundance. **F)** Principal component analysis (PCA) of the five microhabitats with species as variables and abundances correlated to lengths rarefied and non-rarefied (inlet). Space constricted, numbers correspond to species in (Fig. 3)

The principal component analysis (PCA) generated with species as variables and habitats as observations (Fig. 2F), outlined the strength of the different species and their abundance to define each of the partitions in which the intertidal was divided. Amphipod genera *Echinogammarus* and *Orchestia*, together with the arthropod *Anurida maritima* were the endpoints that presented higher positive correlation with the first component (PC1). The occurrence of these species characterized the uppermost belt of the intertidal (yellow area) in Newton's Cove, as they occurred almost exclusively in this zone. In the non-rarefied PCA (Fig 2F, inlet), the weight of *Echinogammarus* sp. as a variable showed that apart from being almost exclusive of this area it was very abundant as well. Similarly, the platyhelminth *Procerodes littoralis* and the polychaete *Capitella capitata*, were very abundant behind boulders in the "Yellow area", but also amongst the little stones and gravel constituting the "Grey area". Notice that smaller individuals and larvae of *Echinogammarus* sp. and *Procerodes littoralis*, were sampled in a lower section of upper intertidal than their adult counterparts.

Concomitantly to the first algal assemblages (Green area) a surge in the relative abundance of another amphipod (*Melita* sp.) and *Littorina* spp. Snails was observed. Different species of small nematodes were present in this habitat as well. Since most species in Newton's Cove, were only observed in the lower part of the intertidal and in modest abundances (Figs. 2C & 2D), the number of vectors with similar size and orientation made them undistinguishable in the PCA (Fig. 2F). Consequently, the relative abundance of each species in the different zones of the intertidal was shown in a heatmap illustrating single extraction occurrence probabilities (Fig. 3); the probability of finding a given species by randomly sampling a single individual in each of the habitats defined in Newton's Cove. This information regarding the location and abundance of the different invertebrate species inhabiting Newton's Cove facilitated the selection of suitable candidates as reservoir/intermediate hosts for histopathological screenings of protist parasites. The small polychaete *Capitella capitata* and the amphipod *Echinogammarus sp*. represented the two most abundant invertebrates observed in the upper part of the intertidal zone in Newton's Cove. Their combined relative abundances in the "yellow area" and "grey area" were greater than the rest of species together if copepods are not considered. Buried in the moist sand, harpacticoid copepods were extremely abundant in the upper intertidal zone but not counted when exceeding the 200 individuals per sample, being therefore excluded from estimations of relative abundance. Gastropods *Littorina* spp. and *Gibbula* spp. were also present in the upper intertidal, but their relative abundance was much lower, 0.18% and 1.11% respectively.

Unlike *Echinogammarus* sp. and *Capitella* sp., the turbellarian *Procerodes* sp. is abundant in upper and lower intertidal zones, so are its eggs and larvae. *Melita* sp. amphipods, halacarid sea-mites, and *Notomastus* sp. polychaetes emerged in less (air) exposed zones of the upper intertidal, with the

first algal assemblages (genera *Blindingia*, *Ectocarpus*, *Ulothrix,* and *Ulva*). The habitats constituting the lower part of the intertidal ("Purple" and "Brown" areas) were substantially richer in number of species, but the relative abundance was reduced at least by an order of magnitude. Gastropods *Littorina obtusata*, *Littorina littorea*, and *Gibbula* sp. were amongst the most abundant species in ponds and behind rocks together with the isopods *Dynamene bidentata* and especially *Jaera ischiosetosa*. Molluscan genera *Patella* and *Epitonium,* in conjunction with the crustacean *Chthamalus* sp. dominated more exposed zones and ridges.

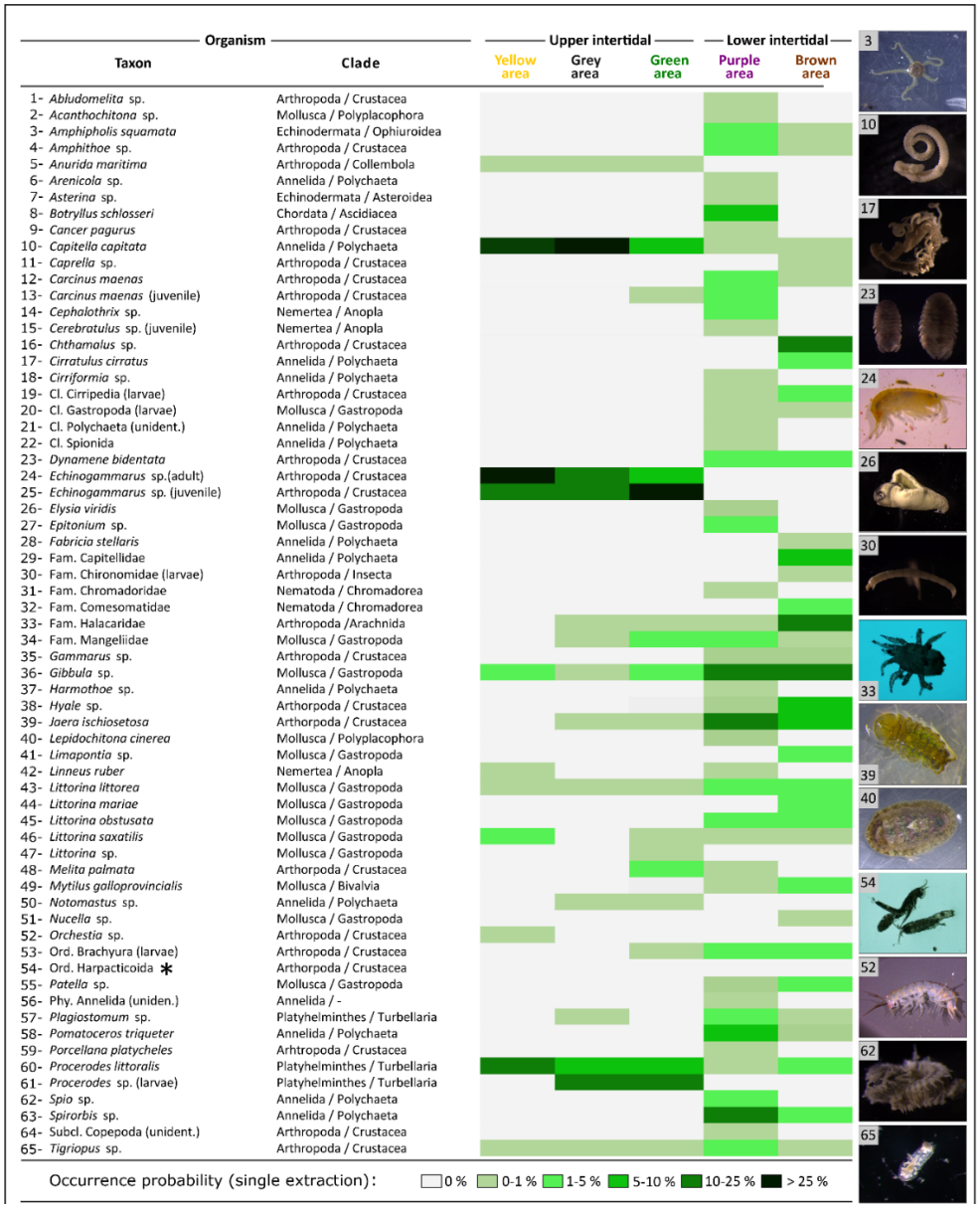

Figure 3: Heatmap indicating the relative abundance of species in each of the five microhabitats defined in Newton's Cove (Fig. 1). Colours indicate the probability of species to be selected after extraction of a single individual, in each of the microhabitats independently. Grey colour indicates 0% probability of being selected when a single individual is randomly extracted from a given microhabitat. A scale of greens shows increasing probability. Sampled organisms are linked to clade and to the same number used in (Fig. 2F). Numbers also correspond to illustrative pictures of some of them in the right margin.

3.2 Histology and pathology of common invertebrate species in Newton's Cove

The most abundant invertebrates identified in Newton's Cove (*Echinogammarus*, *Capitella*, *Procerodes*, and harpacticoid copepods) were processed for histological analysis. Their small body size (0.1 cm – 1.5 cm in length) allowed for whole-body fixation (Fig. 4) in which the structure and position of the different organs and tissues was well retained. This is especially true in the case of amphipods and copepods, in which the exoskeleton, allowed a sagittal sectioning that showed all major organs (Fig. 4A, 4B, 4G & 4H).

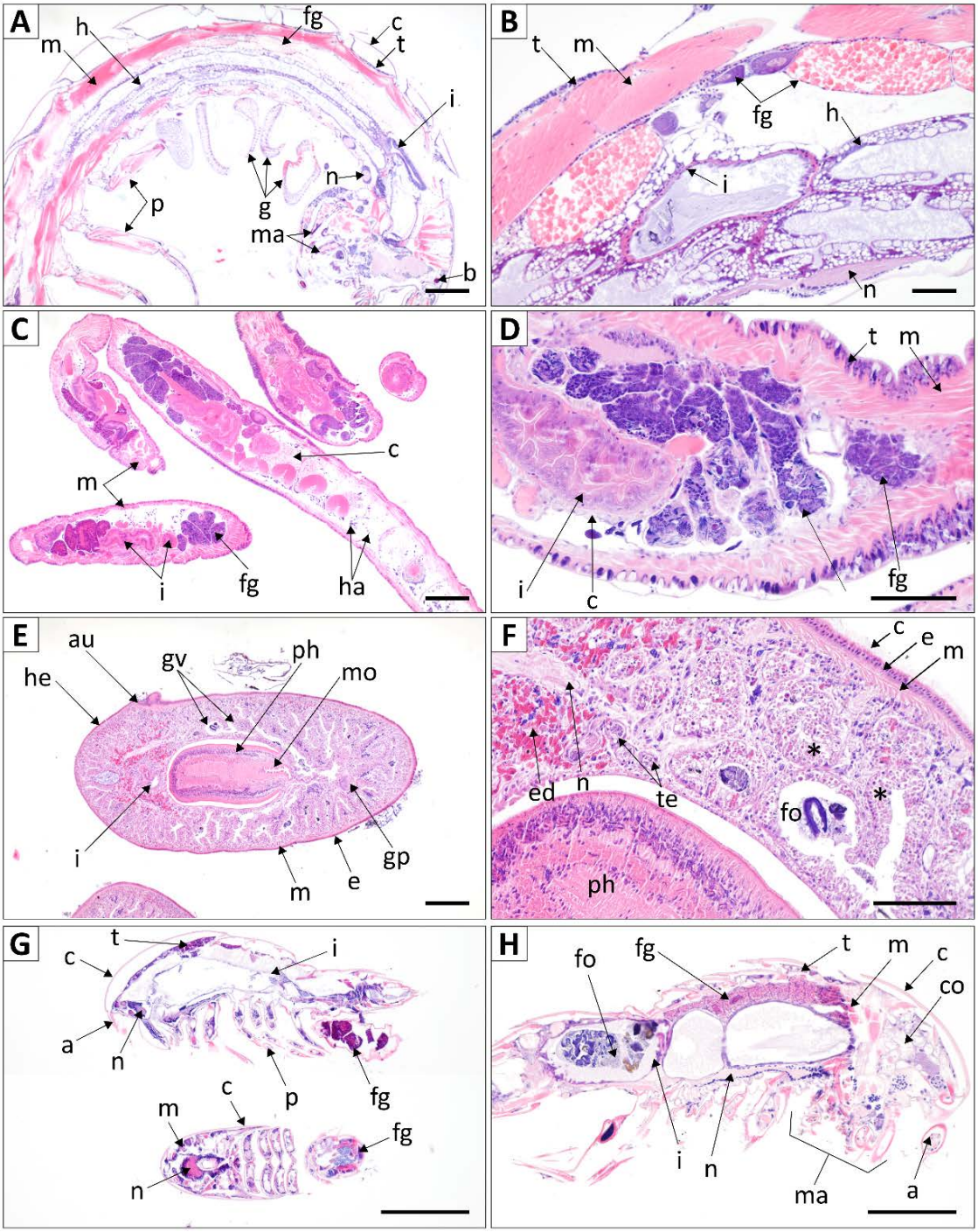

Figure 4: (Preceding page) Histological appearance of *Echinogammarus* sp., *Capitella* sp., *Procerodes* sp., and harpacticoid copepods in (Family Ameiridae). **(A)** Sagittal section of an *Echinogammarus* sp. amphipod, and detail of its main organs and tissues in **(B).** Brain (b), carapace (c), female gonads (fg), gills (g), hepatopancreas (h), gut (i), muscle (m), maxilla and mandible (ma), nerves and ganglia (n), pleopods (p), tegument (t). **(C)** Sagittal and transversal sections of *Capitella* sp. polychaetes and detail of the main organs and tissues **(D)**. Connective (c), female gonads (fg), haemocytes (ha), gut (i), muscle (m). **(E)** Coronal section of a *Procerodes* sp. platyhelminth, and detail of its main organs and tissues **(F).** Auricle (au), cilia (c), epidermis (e), excretory ducts (ed), food (fo), digestive gland (*), genital pore and penis (gp), gastrovascular cavity (gv), head (he), gut (i), mouth (mo), muscle (m), nerves and ganglia (n), pharynx (ph), testis (te). **(G)** Sagittal and coronal sections of the body of a harpacticoid copepod (Fam. Aimeridae), and detail of its main organs and tissues **(H)**. Antennule (a), carapace (c), connective tissue (co), female gonad (fg), food (fo), gut (i), muscle (m), mandible and maxilla (ma), nerves and ganglia (n), pereopods (p), tegument (t). Scale bars = (A, C) 200 µm, (B, E, G) 100 µm, (D, F, H) 50 µm.

Several micro-eukaryotic, bacterial and viral parasites/symbionts were observed either infecting or associated to tissues in the four invertebrates analysed (Fig. 5). Alveolate protists from Phylum Ciliophora, were the most abundant micro-eukaryotes observed in all hosts, followed by gregarines (Phylum Apicomplexa). In *Echinogammarus* sp., ciliates were usually observed attached to pereopods, pleopods, and gills and their incidence varied between 92% and 100% depending on the month. In *Capitella sp*., ciliates were found either attached to the tegument or associated to the gut and its lumen. Its prevalence varied greatly depending on the season, being considerably higher during late summer and autumn (70 - 90%) than in spring and early summer (Fig. 5B). In *Procerodes* sp. and harpacticoid copepods, the prevalence of ciliates was considerably lower (5 - 20%).

Gregarines appeared to be equally abundant in *Procerodes* sp., *Capitella* sp. and *Echinogammarus* sp., with apparent prevalence values ranging between 20% and 35%. Metazoan parasites, including copepods, nematodes and trematodes were almost exclusively observed in *Echinogammarus* sp., the largest of the hosts investigated, especially during July. Microsporidians, a phylum of obligatory intracellular parasites related to fungi, were observed infecting polychaetes and amphipods. In *Capitella* sp., infection was predominantly observed in the gut epithelium, while in *Echinogammarus* sp. the main target tissue was skeletal muscle. During summer months (July, August, and September), up to 15% of the polychaetes and amphipods examined were infected. Haplosporidians and paramyxids, which belong to the rhizarian Phylum Cercozoa, are important parasites of molluscs and crustaceans, respectively. Both were exclusively observed infecting *Echinogammarus* sp. Approximately, a 20% of amphipods collected during June were infected by haplosporidian microcells. Similar infection rates were observed for paramyxid parasites but during late summer (September). Unicellular holozoans were also identified infecting *Echinogammarus* sp. in June. Apart from eukaryotic parasites, bacteria were observed in tissues of all four invertebrate hosts

but were especially abundant in *Procerodes* sp. during late summer and autumn. Histological evidence of viral infections was only noticed in few individuals of genera *Procerodes* and *Echinogammarus*. Significantly smaller in size, copepods were the least parasitized of all the studied hosts. In contrast, amphipods, slightly bigger than *Capitella* sp. and *Procerodes* sp., harboured several clades of important parasites and with a higher prevalence. In fact, several protist lineages causing infections in *Echinogammarus* sp. were not detected histologically in the other hosts.

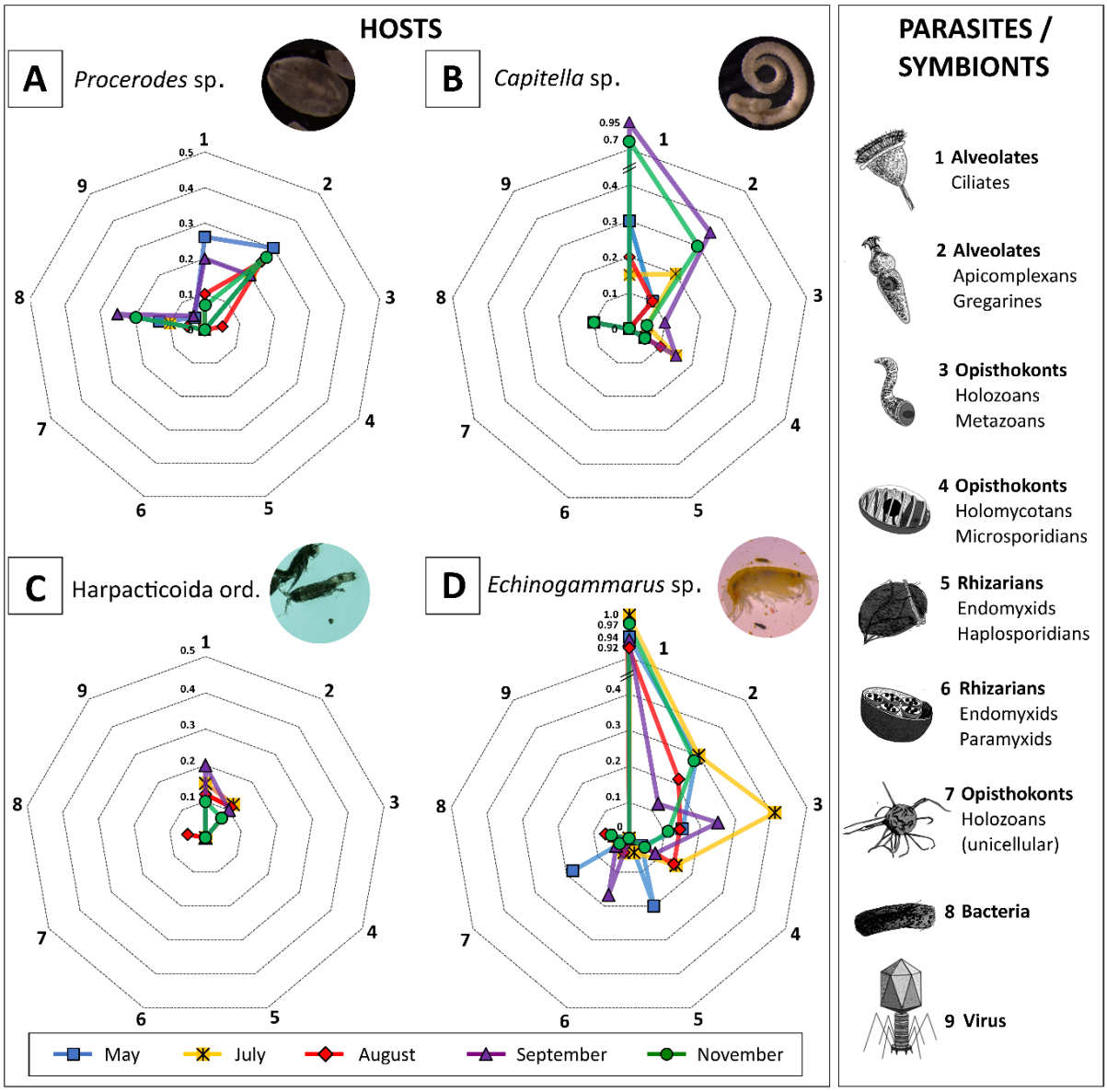

Figure 5: Radar plots showing the incidence of prostist parasites/symbionts in *Procerodes* sp (**A**)., *Capitella* sp. (**B**), harpacticoid copepods (**C**), and *Echinogammarus* sp. (**D**) hosts, collected in Newton's Cove (Weymouth) from June to November (2019). Numbers from 1 to 9 in the radar plots correspond to the parasites/symbionts specified in the box to the right. Values ranging from 0 to 1 in the centre of the plot indicate the incidence of each parasite for the different hosts. In plots corresponding to *Capitella* sp. and *Echinogammarus* sp. the axis is broken at 0.5 (50%) and prevalence values are indicated. In total 400 individuals were analysed, 100 for each host (20 specimens per month).

3.3 Histology, ultrastructure and phylogeny of the main parasites infecting *Echinogammarus* sp. and other amphipods.

Highly abundant in Newton's Cove and profusely parasitized, a full histopathological screening of *Echinogammarus* sp. was conducted periodically (monthly) from April 2016 to September 2017. Additionally, other amphipod genera present in Newton's Cove were also collected and investigated for pathogens and associated micro-eukaryotic parasites for comparison. Ciliates belonging to genera *Isochona*, *Vorticella*, *Cothurnia*, *Gymnodinioides*, *Rhabdostilla*, *Zoothamnium*, *Paracineta*, *Hartmannula*, *Philaster*, or *Uronemella* (according to the metagenomic analysis), were frequently observed attached to pereopods and pleopods (walking and swimming legs) in amphipods. However, they were most common in gills, often penetrating the outer layer of the integument. In rare occasions, ciliates were observed inside gill epithelia as well (Fig. 6A) associated with necrotic gill cells. The frequent occurrence of gregarines, particularly *Heliospora* sp. (according to metagenomic analysis), in the gut lumen of amphipods, did not have any apparent adverse consequences for the host. However, in some cases they proliferated to reach high numbers in the gut (Fig. 6B), with at least one of the life-cycle stages attaching to the gut epithelium; seldom penetrating within the gut-enclosing connective tissue layers.

Parasites belonging to the rhizarian orders Haplosporida and Paramyxida (Fig. 6D & 6E) were commonly observed in *Echinogammarus* sp. and other amphipod genera sampled in Newton's Cove (*Orchestia* and *Gammarus*). These obligatory protist parasites were similar in size to the parasitic microcells in the opisthokont lineages Microsporidia and Filasterea (Fig. 6C & 6F). The size and position of the nucleus, translucency of the cell, and eosinophilia of its cytoplasm, together with cell division and tissue tropism provided a guide for their likely identification (Table 4). The structural damage to the tissues and evident host reactions elicited by protists in these four clades (Haplosporidia, Paramyxida, Microsporidia, and Filasterea) was evident, often including inflammatory responses, melanin encapsulations, and granuloma formations (Table 4; Pathology). Roughly doubling the size of these microcells, a parasitic dinoflagellate was observed infecting *Gammarus* sp. amphipods (Fig. 6G). Parasite cells crowded the haemolymph and haemal sinuses, but no apparent host reaction was noticed. Oomycete-like parasite cells (Fig. 6H) were exclusively observed in the female eggs of genera *Echinogammarus* and *Gammarus*, halting their development, with pluripotential cells appearing necrotised and unviable.

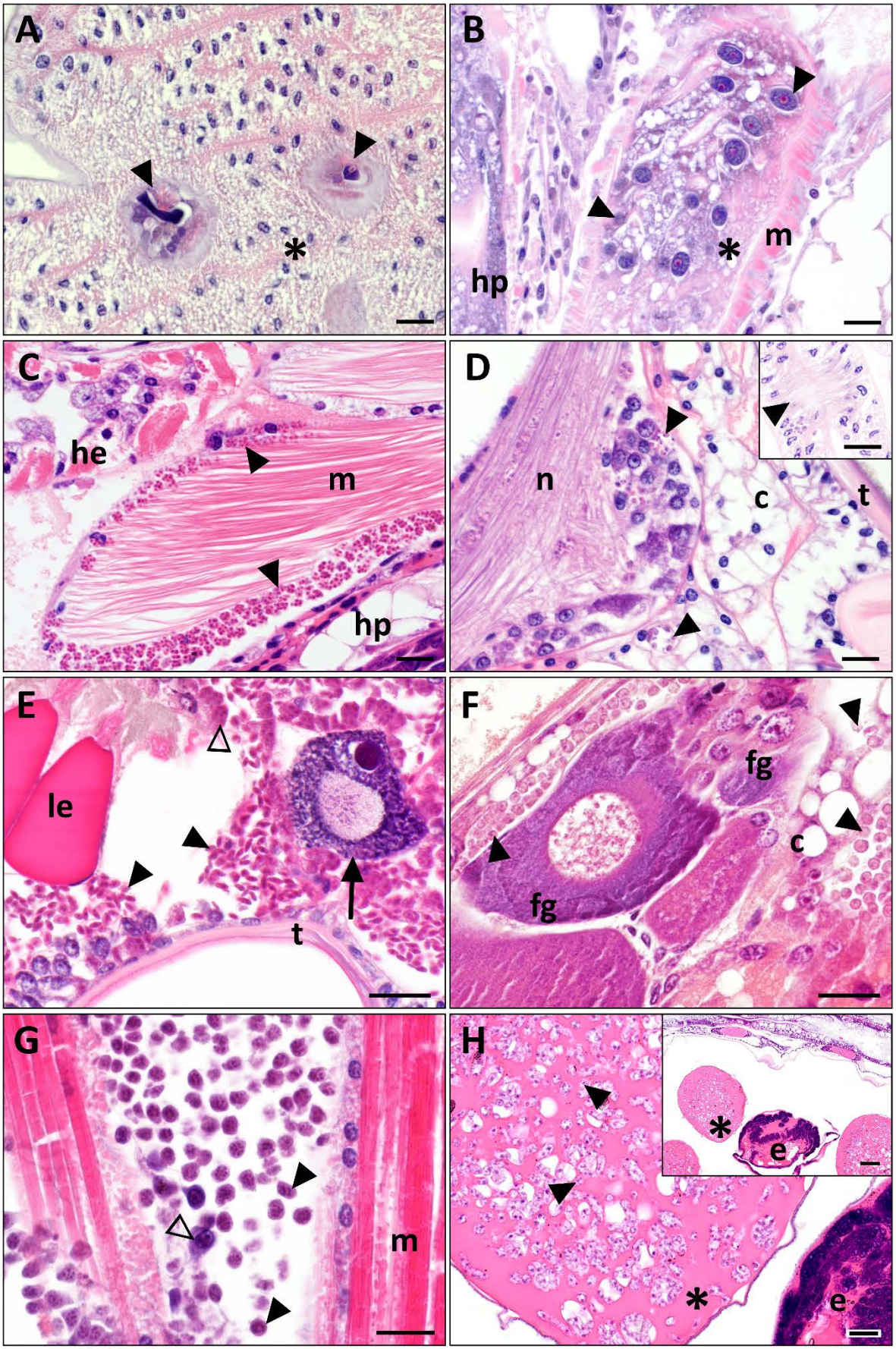
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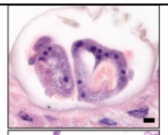
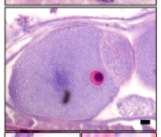
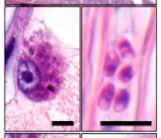
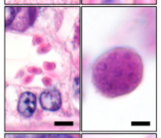
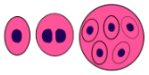
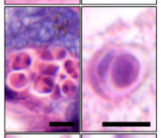
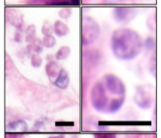
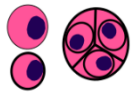
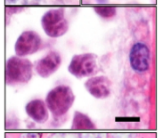
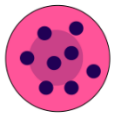
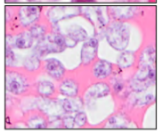
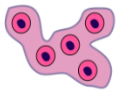
Figure 6: (Preceding page) Light microscopic images of parasitic and endosymbiotic protists in amphipod hosts (H & E stained). **(A)** Two unidentified ciliates (arrowheads) embedded within *Echinogammarus* sp. gill tissue (asterisk). **(B)** Gregarines (arrowheads) in the gut (asterisk) of Orchestia sp. Muscles around the gut (m) and hepatopancreas (hp) are indicated. **(C)** Microsporidia (arrowheads) infecting the skeletal muscle (m) in *Echinogammarus* sp. hosts. Heart (he) and hepatopancreas (hp). **(D)** Microeukaryotic parasites (arrowheads) belonging to order Paramyxida (Cl. Ascetosporea) infecting nervous tissues (n), tegument (t) and associated connective tissues (c) in *Echinogammarus* sp. **(E)** Haplosporidian parasite (Cl. Ascetosporea). Unicellular stages (black arrowhead) and plasmodia (empty arrowhead) were observed congesting the haemolymph and tegument (t) of *Orchestia* sp. The normal conformation of sensory cells (arrow) was compromised in a completely substituted tegument. Eye lenses (le). **(F)** Filasterean parasite *Txikispora philomaios* (arrowhead) congesting the haemolymph and connective tissues associated to the female gonads (fg) of *Orchestia* sp. **(G)** Protist cells belonging to Order Syndiniales (Phylum Dinoflagellata) (arrowhead) congest the haemal sinuses in *Gammarus* sp. Haemocytes (empty arrowhead) and skeletal muscle (m). **(H)** Oomycete-like organsims forming hyphae (arrowheads) infect the eggs (*) contained within the female marsupium of an *Echinogammarus* sp. individual; halting their development. In the inlet an infected and inviable egg (*) and a healthy looking one (e). Scale bars for A, B, C, D, E, F, G, and H = 20 µm. Inlet in D = 50 µm. Inlet in H = 100 µM.

Apart from characteristic morphological traits and differences in tissue tropism, each of the protist parasites/endosymbionts presented a marked temporal variability (Table 4; Prevalence), which assisted on its identification. In most cases, identification using light microscopy was confirmed by Transmission-Electron-Microscope (TEM) analysis (Fig. 7), and/or molecular techniques. The phylogeny of filasterean and haplosporidian parasites infecting amphipods is described in Chapters 2 and 3 respectively, which investigate the diversity of the clade to establish *Haplosporidium orchestiae* n. sp., *Haplosporidium echinogammari* n. sp. (Urrutia et al. 2019), and *Txikispora philomaios* n. sp. n. gen (Urrutia et al. 2021). All paramyxid microcells observed to infect amphipods appear to be closely related to *Paramarteilia orchestiae* (Fig. 8), a common parasite of talitrid amphipods also found in bivalve molluscs. The DNA sequenced from microsporidians infecting amphipod genera *Echinogammarus*, *Orchestia* and *Gammarus* showed that at least two different species in the widely distributed amphipod infecting genus *Dictyocoela* were present in Newton's Cove (Fig. 9). The dinoflagellate observed infecting *Gammarus* sp. was sister to the copepod-infecting genus *Syndinium,* which, together with *Hematodinium* sp. *Amoebophyra* sp. *Duboscquella* sp. and *Ichthyodinium* sp. constitute the dinoflagellate Order Syndiniales (Fig. 10).

Table 4: Identification key for the main protist parasites infecting amphipod genera *Echinogammarus*, *Orchestia*, and *Gammarus* from coastal ecosystems in southwest UK. Parasitic lineages match their known amphipod host(s), a picture, and a schematic diagram of unicellular and multicellular stages. The average size of parasitic cells measured in unikaryotic stages (*), the morphology, tissue trophism, and pathology are also specified in conjunction with a measurement of relative prevalence.

Parasite / Symbiont	Clade	Host	Picture	Schematic diagram	Size	Morphology	Tissue tropism and patholohy	Prevalence
Ciliophora	SAR → Alveolata	*Echinogammarus* sp. *Orchestia* sp. *Gammarus* sp.			Length = 24.34 ± 7.83 µm Width = 16.42 ± 3.39 µm	Round, elongated or cup-shaped cells surrounded by cilia. They posses a long macronucleus and often numerous small micronuclei	Often attached to host gills and carapace, ciliates penetrate the tegument cuticle. In few occasions ciliates have been observed in association to disrupted gill tissue as well. However, they appear to be predominantly epibiotic in amphipods.	Between 70% and 100% of the hosts are infected. Prevalence is higher during summer.
Gregarinasina	SAR → Alveolata → Apicomplexa	*Echinogammarus* sp. *Orchestia* sp. *Gammarus* sp.			Length = 30.77 ± 13.70 µm Width = 18.12 ± 7.68 µm	The granular and haematoxilinophilic macrogamont is possibly the most easily identifiable of the stages in the complex life cycle of these protists	Usually free in the lumen of the digestive, they can also penetrate inside and between intestine cells, When present in high numbers, gregarines can affect the normal conformation of the gut and associated tissues.	Between 30% and 60% in *Echinogammarus* sp. Prevalence varies between 15% and 40% in *Orchestia* sp. and *Gammarus* sp.
Microsporidia	Opisthokonta → Holomycota	*Echinogammarus* sp. *Orchestia* sp. *Gammarus* sp.			Length = 2.76 ± 0.31 µm Width = 1.76 ± 0.12 µm	Small and oval cells with a thick wall, usually forming compact groups inside host cells. The presence of a big vacuole opposed to the nucleous, gives them a refractile appearance.	Microsporidian parasites are predominantly observed in skeletal muscle, where infection causes necrosis and disruption of fibrillar cells. Tegument, connective, gills, and ovaries are important target tissues as well.	Up to 20% in *Echinogammarus* sp. A 30% in *Orchestia* sp., and 45% in *Gammarus* sp. Highest levels occur during summer (July & August).
Haplosporidia	SAR → Rhizaria → Ascetosporea	*Echinogammarus* sp. *Orchestia* sp. *Gammarus* sp.			* Length = 3.01 ± 0.62 µm Width = 1.90 ± 0.28 µm	Multinucleated plasmodia are charcateristic of this protist order. The non-walled trophonts have a small central nucleous and a stronlgy eosinophilic cytoplasm.	Infection usually develops in connective tissues, especially those associated to digestive and tegumental glands. Following, the haemolymph is colonized, facilitating infection of other organs such as hepatopancreas and intestine.	Up to 75% of *Echinogammarus* sp. individuals can be affected during late May and June. In *Gammarus* sp. and *Orchestia* sp. values are lower (20%).
Paramyxida	SAR → Rhizaria → Ascetosporea	*Echinogammarus* sp. *Orchestia* sp. *Gammarus* sp.			* Length= 3.32 ± 0.39 µm Width= 3.14 0.38 µm	A round cell with a small nucleus is contained within a nucleated cell. A "cell within cell" scheme characteristic of this order. The nuclei of the inner and outer cell appear as opposed to each other.	Predominantly associated to nerves and ganglia, the structure and viability of neurons is often compromised. Usually, connective tissues around the nerves, tegument, gills, and female ovaries are affected in infected individuals.	Prevalence peaks in late summer (September), when about 25% of *Echinogammarus* sp., 20% of *Gammarus* sp., and 15% of *Orchestia* sp. ind. appear infected.
Filasterea	Opisthokonta → Holozoa	*Echinogammarus* sp. *Orchestia* sp.			* Length = 2.36 ± 0.23 µm Width = 1.94 ± 0.21 µm	The slightly ameboid and virtually round cells may be walled or not. In dividing stages of the parasite, three walled cells are evident within a non-nucleated walled parent cell.	Infection develops inside host haemocytes and connective tissues associated to the tegument. In heavily affected individuals haemolymph appears completly congested, and other organs might be affected. Gonads have been observed to be infected in otherwise healthy individuals.	In *Echinogammarus* sp., infection peaks during May (64%). The rest of the year infection is exclusively observed in gonads. In *Orchestia* sp. prevalence is lower (~10%)
Dinoflagellatea	SAR → Alveolata	*Gammarus* sp.			Length = 6.51 ± 0.37 µm Width = 5.60 ± 0.46 µm	Weakly stained, the nucleous is not evindent in ameboid trophonts (only stage observed). In contrast up to 10 haematoxilinophilic bodies are evident inside cells.	In *Gammarus* sp. the haemolymph seems to be the only target tissue, although it is densely congested by ameboid trophonts. No evidence of host reaction such as melanization or granuloma formation has been observed.	Only observed in *Gammarus* sp. individuals collected in Tamar estuary. Prevalence values were as high as 30% in April.
Oomycetes	SAR → Stramenopiles	*Echinogammarus* sp. *Gammarus* sp.			Length = 2.99 ± 0.43 µm Width = 2.39 ± 0.37 µm	Virtually round cells with a central nucleus and a weakly stained cytoplasm. Individual cells appear imbedded in a matrix formed by oogonial and hyphae-like structures.	In amphipods, infection by oomycetes has only been observed in eggs / larval stages. Affected larvae appear necrotic and their development is halted.	About 5% of individuals of *Echinogammarus* sp. and *Gammarus* sp. collected during March.

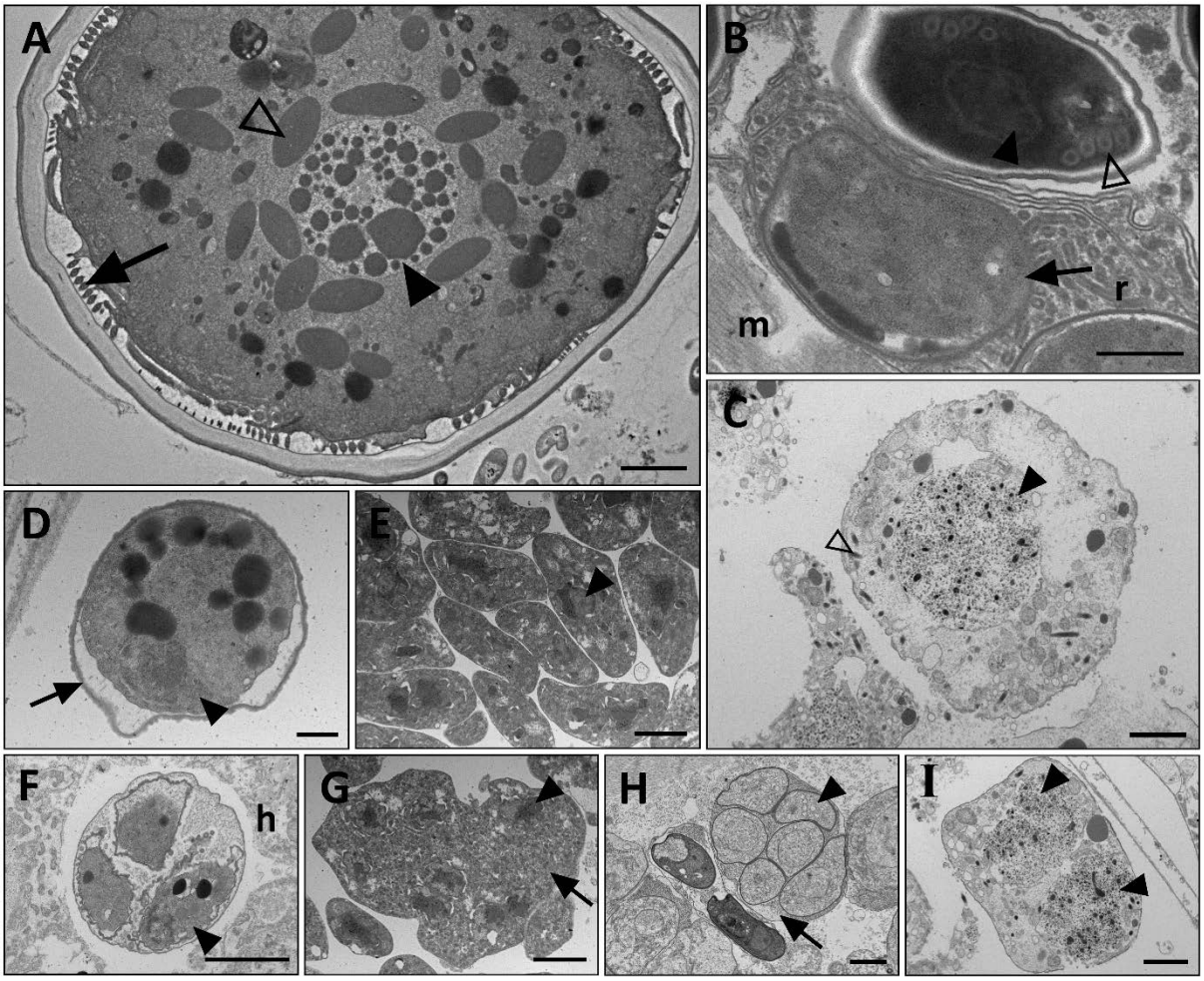


Figure 7: Transmission Electron Microscope (TEM) micrographs of amphipod associated protist parasites/symbionts. **(A)** Ectoparasitic ciliate attached to the gills of *Echinogammarus* sp. A central macronucleus (arrowhead), trichocysts (empty arrowhead) and cilia (arrow). **(B)** Late stage sporoblast (arrow) and spore illustrating the polar filament (empty arrowhead) and nucleus (arrowhead). **(C)** Unikaryotic trophont of *Syndinium* sp.infecting *Gammarus* sp.haemolymph. Nucleus (arrowhead) and trichocysts (empty arrowhead). **(D)** Walled unikaryotic stage of the filasterean *Txikispora philomaios* infecting the haemolymph of *Orchestia* sp. Nucleus (arrowhead) and cell-coat (arrow) **(E)** Unikaryotic and dikaryotic stages of *Haplosporidium orchestiae*. Nucleus (arrow). **(F)** Divisional stage of *T. philomaios* bearing three walled daughter cells (arrow) inside a host haemocyte (h).**(G)** Multinucleated plasmodium of *H. orchestiae* (arrow) bearing up to 8 dividing nuclei (arrowhead). **(H)** Spores and meront of *Dictyocoela* sp. infecting *Echinogammarus* sp. **(I)** Dikaryotic trophont of *Syndinium*sp. Nuclei (arrowheads). Scale bars = A, B, C, E, F, G, H, I = 2 µM, and D = 500 nm.

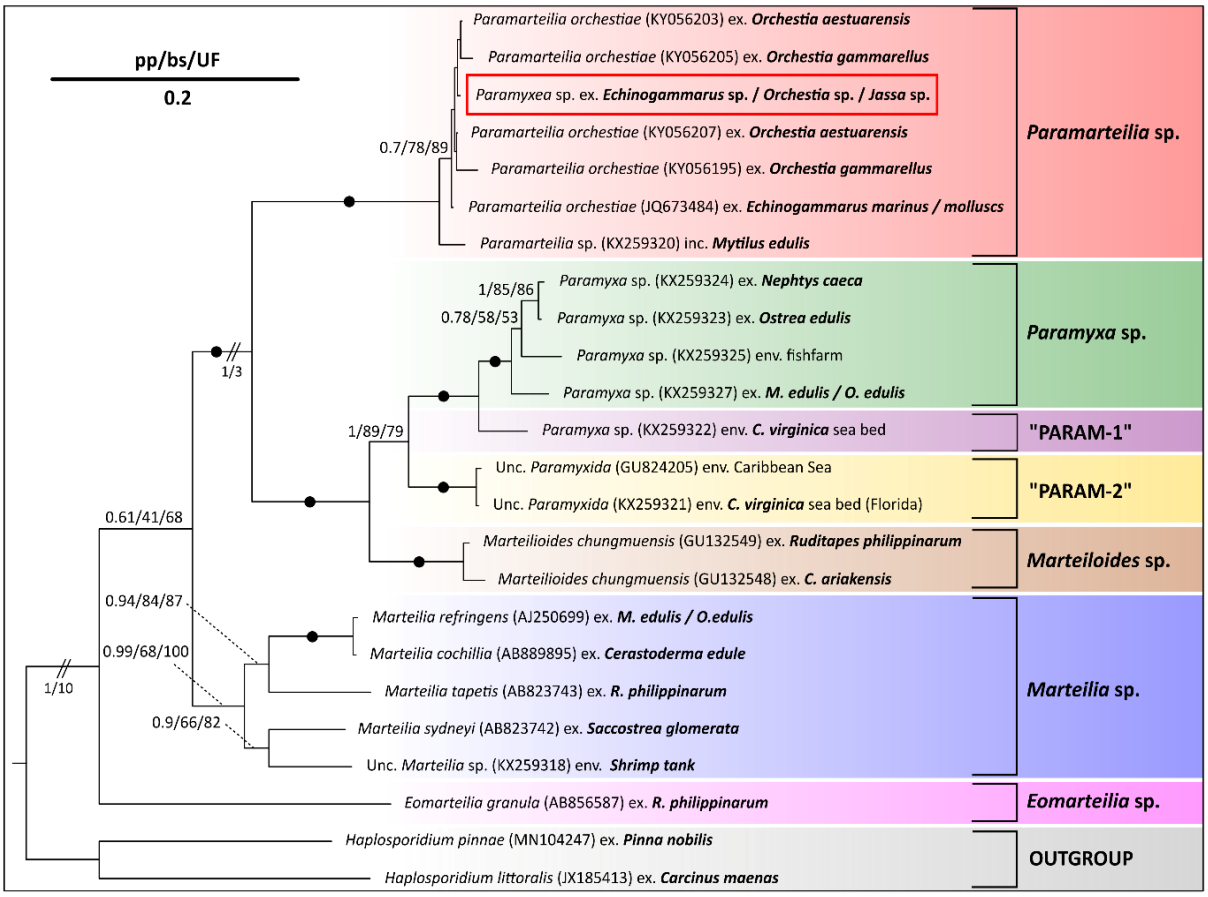


Figure 8: Bayesian phylogenetic analysis of the 18S rRNA gene, recognizes the paramyxean parasite infecting amphipod genera *Echinogammarus*, *Orchestia*, and *Jassa*, as *Paramarteilia orchestiae* (red rectangle). The alignment included a 385 bp fragment (V6 - V8 region) of *P. orchestiae* infecting amphipods from Southwest England and complete (or partial) 18S rRNA from *P. orchestiae* in other studies and amphipod genera. Additionally, the tree included the rest of paramyxid species, their GenBank reference, and the principal but not necessarily the only host. Uncultured sequences are matched to the environment from which they were sampled. Branch support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap support (UF) from IQ-TREE, respectively. Nodes with values (> 0.95 pp, 95 bs, > 95 UF) are represented by a black dot on the branch.

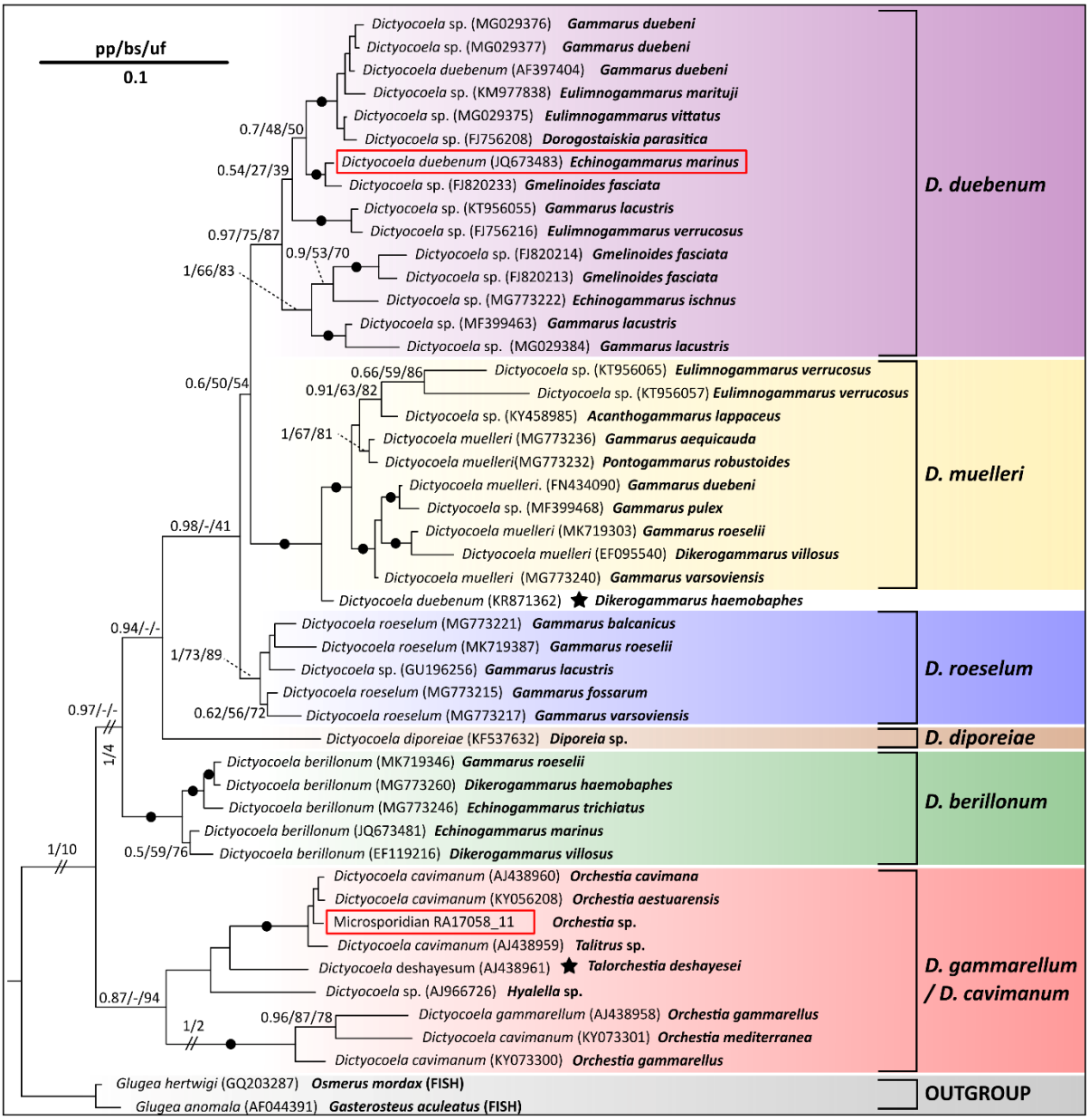


Figure 9: Bayesian phylogenetic analysis of the 18S rRNA and ITS1 genes recognizes the microsporidian parasite infecting *Echinogammarus* sp. as *Dictyocoela duebenum* and that in *Orchestia* sp. as *Dictyocoela cavimanum* (red squares). The alignment included a 900 bp fragment of each *Dictyocoela* spp.infecting amphipods from Southwest England and complete (or partial) 18S rRNA and ITS-1 genes from existing *Dictyocoela* spp. with their Genbank reference, and the principal but not necessarily the only host. A star ascribed to few *Dictyocoela* spp. indicates that they were possibly misidentified in previous studies. Branch support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap support (UF) from IQ-TREE, respectively. Nodes with values (> 0.95 pp, 95 bs, > 95 UF) are represented by a black dot on the branch. The outgroup is constituted by two species belonging to related microsporidian genus *Glugea*, parasitic in osteichthyes.

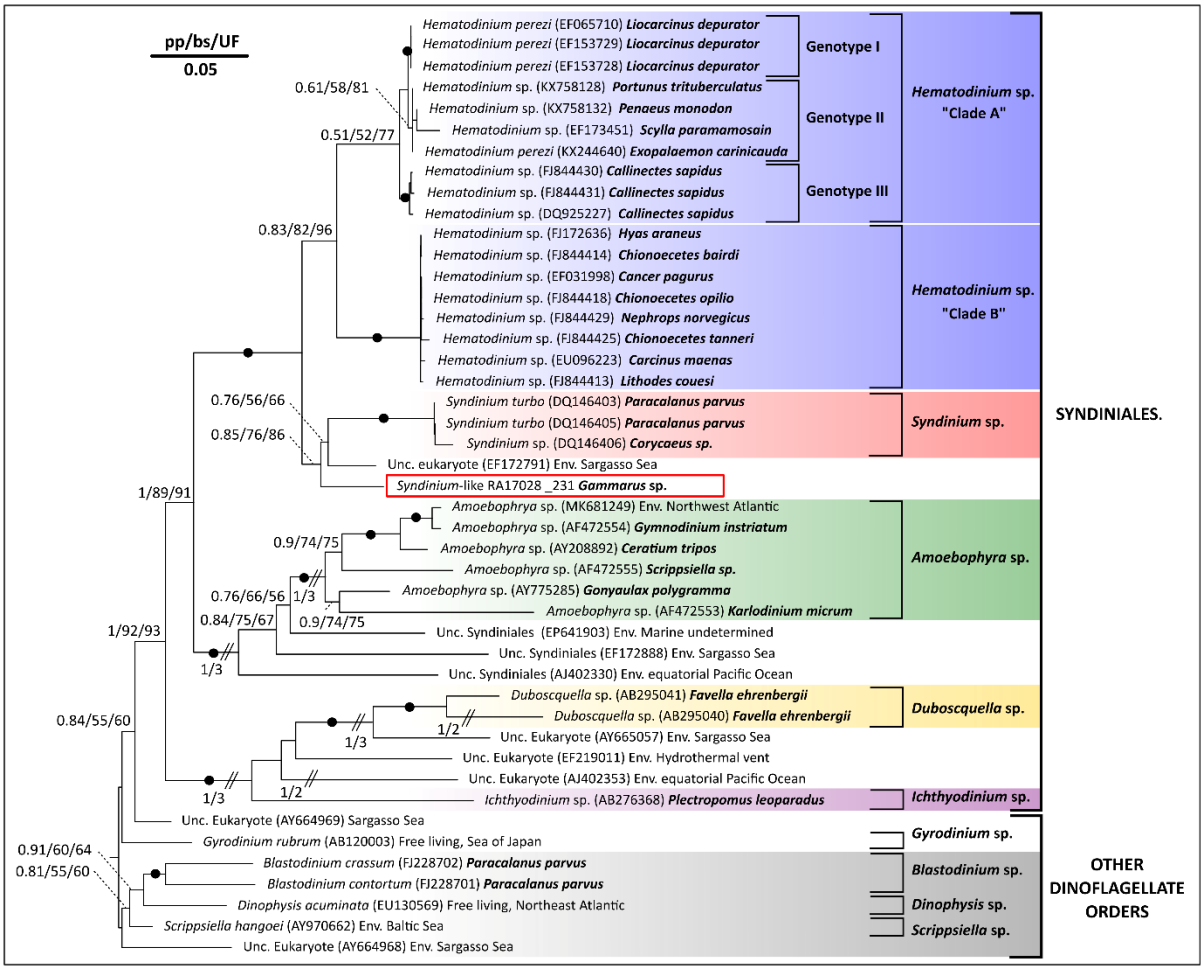


Figure 10: Bayesian phylogenetic analysis of the 18S, ITS1, 5.8S, and ITS2 rRNA genes indicated that the dinoflagellate parasite infecting *Gammarus* sp. (red rectangle) was related to *Syndinium turbo*. The alignment included a 603 bp fragment (ITS1, 5.8S, and ITS2) of the syndinid infecting *Gammarus* sp. in the Tamar estuary together with 18S, ITS1, 5.8S, and ITS2 rRNA genes of other genera in Syndiniales and Dinoflagellate orders with their Genbank reference and the principal but not necessarily the only host. Branch support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap support (UF) from IQ-TREE, respectively. Nodes with values (> 0.95 pp, 95 bs, > 95 UF) are represented by a black dot on the branch.

Concomitant with parasitic protists, other micro- and macro-eukaryotic pathogens, and non-eukaryotes (bacteria, virus) were also monitored as possible vectors or triggers of co-infection (Fig. 11). Flatworms (Phylum Platyhelminthes) and roundworms (Phylum Nematoda) were often observed associated to amphipod tissues. Digeneans (Trematoda; Platyhelminthes) were almost exclusively observed inbedded within the skeletal muscle of amphipods, interfering with the normal structure, and functioning of the muscle fibres. Usually, they elicited a strong inflammatory response and permanent encapsulation in melanin. Nematodes were usually observed in connection to gills and tegument, very rarely inside tissues (Fig. 11B). The connective tissue-infecting microsporidian *Dictyocoela cavimanum* (Fig. 11C) was similar in size to parasitic fungi (Fig. 11D), that also proliferated in connective tissues, integument, hepatopancreas and gut. Fungal infections elicited a systemic host response constituted by numerous granulomas and melanized encapsulations that rarely occurred in sister clade Microsporidia, especially in *D. duebenum* infections. The occurrence of bacteria and viruses was usually noticed in gills (Fig. 11E & 11F). The presence of granular basophilic masses (bacteria) and abnormally large eosinophilic nuclei (viruses) was in some cases associated to cell apoptosis and mild localized tissue disruption.

3.4 Temporal variation of amphipod-infecting parasites in Newton's Cove

Ciliophora and Gregarinasina were the two most prevalent protist clades occurring not only in *Echinogammarus* sp. but in *Gammarus* sp., and *Orchestia* sp. as well (Fig. 12). Prevalence of ciliates in amphipods fluctuated between 70% and 100%, with higher values during summer. The presence of gregarines in the gut of *Echinogammarus* sp. fluctuated between 22% and 62%, forming a saw-like temporal distribution pattern. The prevalence of the apicomplexan was lower in *Gammarus* sp. and *Orchestia* sp., with 10% to 40% of the examined individuals affected. Obligate protist parasites from clades Haplosporidia and Filasterea infected a very high proportion of *Echinogammarus* sp. individuals sampled in Newton's Cove (75% and 64%, respectively) during spring. However, these peaks of infection were short-lived. During 2016, the peak of haplosporidian infection occurred in June, when the filasterean infection had already decreased considerably. The following year (2017) the presence of filasterean parasites was detected from March, as mild infections in the testes of *Echinogammarus* sp. males. However not a single systemic infection was detected until May, co-occurring with a still increasing peak of haplosporidian infection. Although the number of individuals analysed and the sampling frequency was considerably lower for the other two amphipod genera, similar haplosporidian and filasterean infection rates were not detected. Despite not having a seasonal surge of haplosporidian infections, microcells of this clade were identified in approximately a 10% of the individuals of *Gammarus* sp., throughout the year.

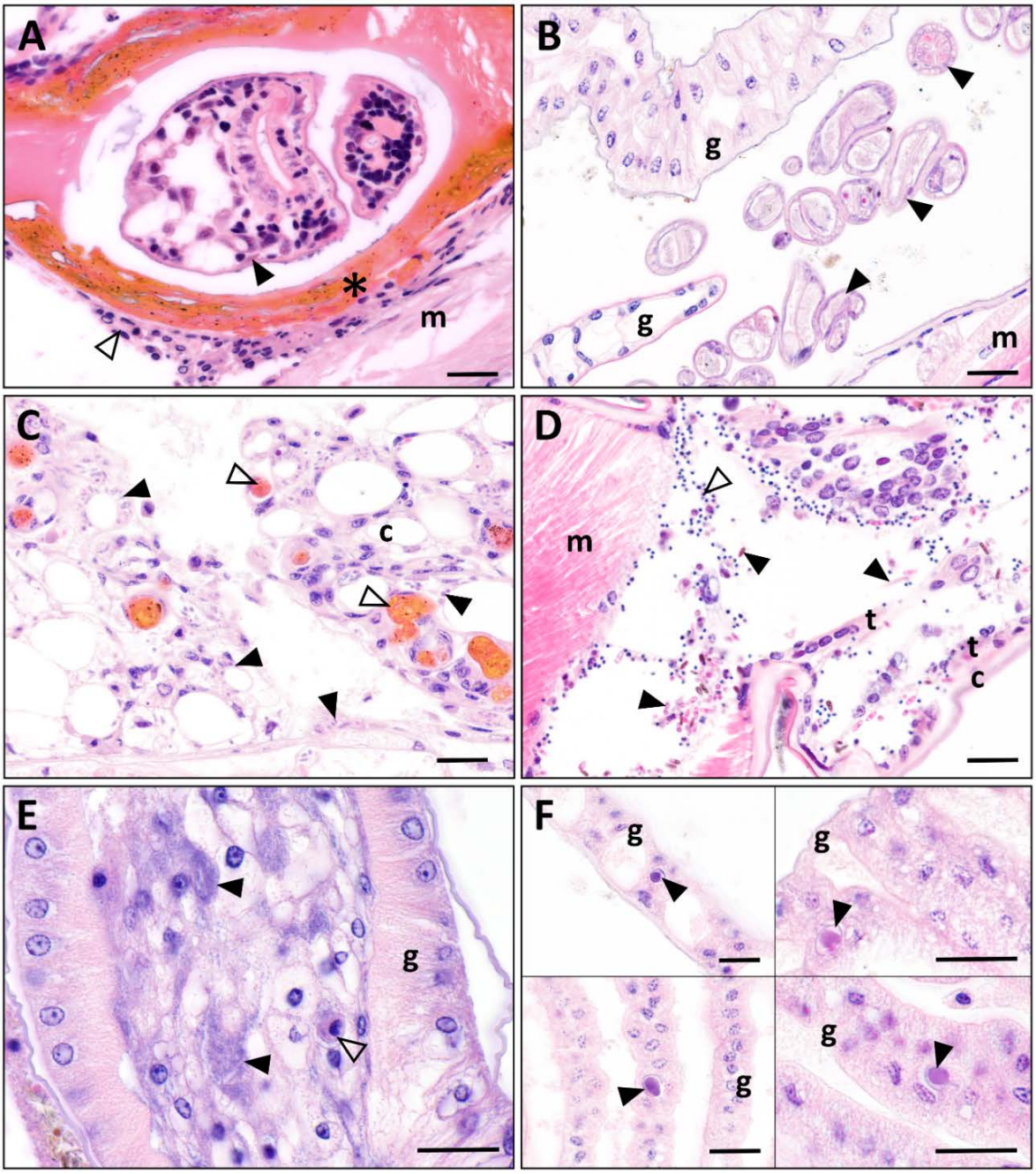


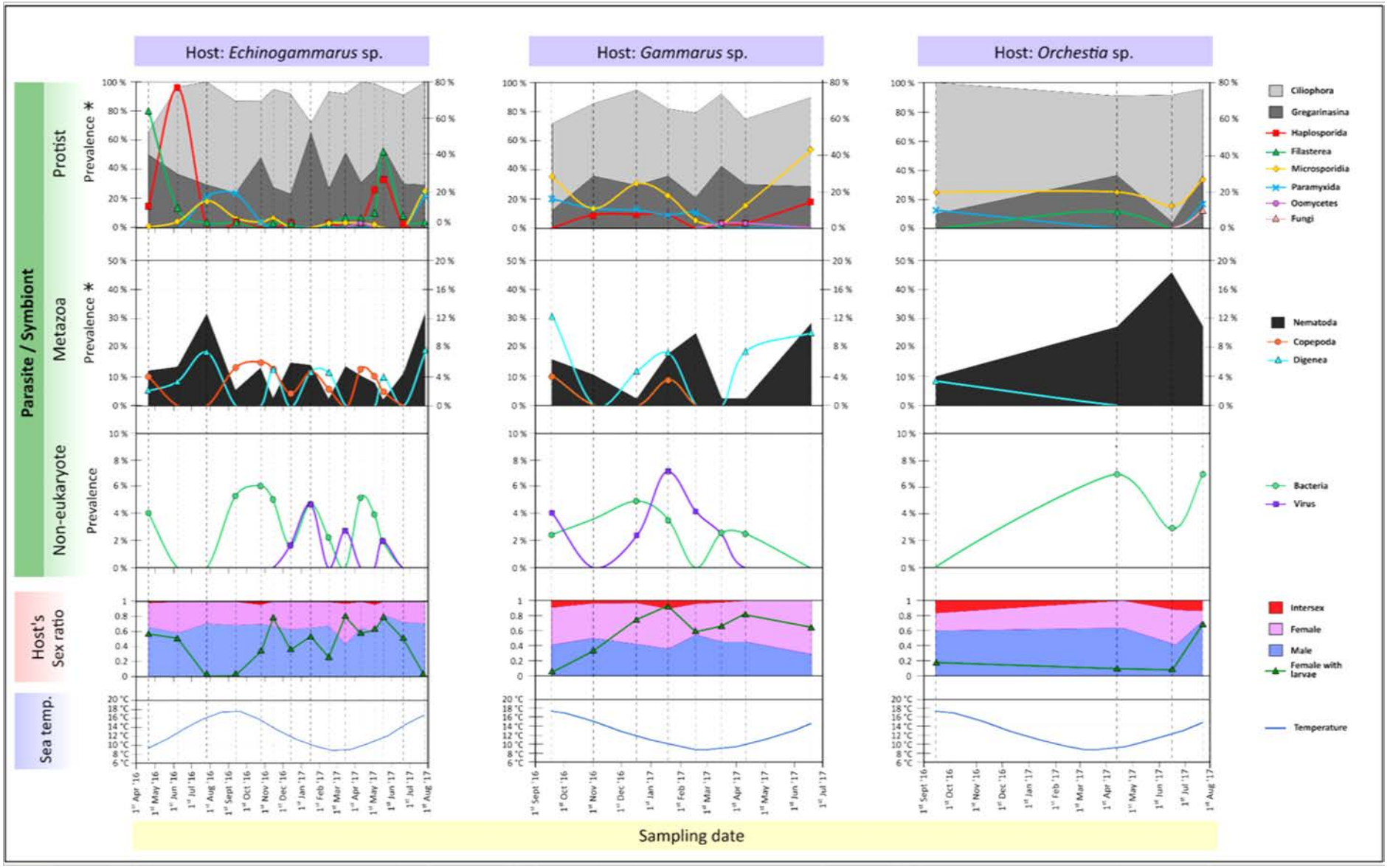
Figure 11: Light microscopic images of eukaryotic and non-eukaryotic parasites infecting amphipod hosts (H & E stained). **(A)** Digenean trematode (Phylum Platyhelminthes) (arrowhead) infecting the skeletal muscle (m) of an *Echinogammarus* sp. host is encapsulated and melanized (*) by host inflammatory cells (empty arrowhead). **(B)** Roundworms (Phylum Nematoda) (arrowheads) are often observed attached to gills (g) and tegument in amphipods **(C)** The microsporidian *Dictyocoela cavimanum* (arrowhead) is frequently observed infecting connective tissues (c), especially those associated to midgut and hepatopancreas, eliciting a strong host reaction including melanisation and formation of granuloma (empty arrowhead). **(D)** A fungal parasite with its hyphae and spores (arrowhead) infects the haemal sinuses, connective, tegument (t), and muscle fibres (m) in a *Echinogammarus* sp. host. **(E)** A mass of Bacteria (arrowhead) infects the connective tissue separating gill lamellae (g) in *Orchestia* sp. Host haemocytes (empty arrowhead). **(F)** Evidence of viral infection in *Echinogammarus* sp. gills (g). Infected gill nuclei (arrowhead) appear significantly enlarged with a basophylic halo in the periphery (nuclear chromatin) and a more eosinophilic mass in the centre (viral DNA). Scale bars for A, B, C, D, E, and F = 20 µm.

Microsporidians appeared all year long infecting the skeletal muscle and connective tissues of the three amphipod genera investigated. The most susceptible amphipod genus appeared to be *Gammarus* sp., with infection prevalence ranging between 5% and 45%. In amphipods sampled from the upper part of the intertidal (Yellow area), microsporidians infected approximately a 20% of the *Orchestia* sp. population throughout the year. Meanwhile, similar values were only observed in *Echinogammarus* sp. during August. Occurring during late summer as well, infections by paramyxids in *Echinogammarus* sp., unseen during the rest of the year, surged, coinciding with the peak of microsporidian presence. While paramyxean parasites disappeared from November to July in *Echinogammarus* sp., infections persisted during all autumn and winter in *Gammarus* sp., with a relative prevalence in the population of around 10%. Short-lived (small) peaks of oomycete-caused infections affecting around a 5% of *Echinogammarus* sp. and *Gammarus* sp. individuals occurred during early spring (March and April 2017). Parasitic fungi were observed infecting about 5% of *Orchestia* sp. individuals in August 2017.

Metazoan parasites occurred in all amphipod genera examined (Fig. 12). Nematodes, which predominantly appeared attached to carapace and gills, were the most abundant, alternatingly appearing during the year in a saw-like crest shadowing that of gregarines. Their prevalence ranged between 5% and 45%, being highest in *Orchestia* sp. Bacterial infections, predominantly noticed in gills, were present in all three amphipod genera, with up to 7% of the *Orchestia* sp. population affected in spring/summer. Histological evidence of viral infections was observed in *Echinogammarus* sp. and *Gammarus* sp., mostly during winter and early spring.

Variations in the male/female ratio and the incidence of intersex individuals were also analysed, together with seasonal seawater temperature changes, and matched to the prevalence of protist and non-protist parasites in Newton's Cove (Fig. 12). The proportion of female amphipods bearing larvae in the pouch, a ventrally located marsupium-like structure, was also considered. The highest level of intersex individuals was observed in genus *Orchestia,* with roughly a 15% of the population presenting this condition in three of the four samplings conducted

Figure 12: (Next page). Temporal variation in the prevalence of protist parasites infecting amphipod genera *Echinogammarus*, *Orchestia*, and *Gammarus* in Newton's Cove (Weymouth) from April 2016 to August 2017. The incidence of protist parasites/symbionts is shown in the three upper graphs and matched to metazoan and non-eukaryotic parasites identified in each amphipod host. Additionally, the prevalence values are coupled to host's sex ratio, number of females bearing larvae, and intersex ratio for each of the samplings. The three bottom graphs indicate the seawater temperature in Newton's Cove measured by a permanently deployed buoy (Cefas). The discontinuous lines traversing the five graphs for each of the amphipods investigated indicate the sampling dates. The prevalence of parasites is shown in the Y axis, which in the first two graphs has two different scales. The one in the left corresponds to organisms whose prevalence is indicated by a coloured area (Ciliophora, Gregarinasina, and Nematoda) and the scale to the right is used for the rest.


Host: Echinogammarus sp.
Host: Gammarus sp.
Host: Orchestia sp.
Parasite / Symbiont
Protist
Metazoa
Non-eukaryote
Host's Sex ratio
Sea temp.
Prevalence *
Prevalence
Ciliophora
Gregarinasina
Haplosporida
Filasterea
Microsporidia
Paramyxida
Oomycetes
Fungi
Nematoda
Copepoda
Digenea
Bacteria
Virus
Intersex
Female
Male
Female with larvae
Temperature
Sampling date

The proportion of intersex amphipods was also considerable in *Gammarus* sp., especially during January and February, when they represented a 12% of the population. In contrast, the occurrence of intersex individuals is almost anecdotic in *Echinogammarus* sp. Regarding the male/female ratio, it was rather consistent for the three amphipod genera studied. The proportion of males is higher in the two amphipod genera inhabiting the upper zone of the intertidal. In contrast, females outnumbered males in the lower intertidal amphipod genus *Gammarus*. The number of larvae-bearing females appeared to increase in November and remained high until late summer (August, September), when the number of females bearing larvae was virtually inexistent in *Echinogammarus* sp. and *Gammarus* sp. The distribution curve outlining the number of larvae-bearing females matched, not only the decrease in sea-water temperatures in Newton's Cove, but also a surge of microsporidian and paramyxid parasites, at least in *Echinogammarus* sp.

The influence of each parasite and its abundance (as prevalence), on the temporal variability observed between the three different amphipod genera was investigated by a PCA (Fig. 13). The variation explained by the two main components was 76.12%. Infection by parasitic filastereans was the endpoint that presented higher correlation (-0.28) with negative values of the main component (PC1) together with gregarines (-0.27) and haplosporidian parasites (-0.20). In turn, microsporidian and paramyxid infections (0.22 and 0.17 respectively) were positively correlated. The second component (PC2) was positively correlated with haplosporidian infections (0.42) and ciliate occurrence (0.204), and negatively correlated with oomycetes (0.071). Based on these variables, the area constituted by histopathological observations on *Echinogammarus* sp., fell predominantly in the negative partition of the main axis, driven by short-lived filasterean and haplosporidian infections during spring, and higher prevalence of gregarines. In contrast, the areas of *Gammarus* sp. and *Orchestia* sp. were largely constituted by observations falling in the positive flank of the main axis, as a result of continued microsporidian and paramyxid presence throughout the year. Few observations laid within the positive segment of the second axis, which showed that during June the prevalence of haplosporidians and ciliates was higher in all three amphipod species. Most observations pertaining to autumn and winter fell near the intersection between axes, weakly characterized by a higher prevalence of oomycete, bacterial and viral infections.

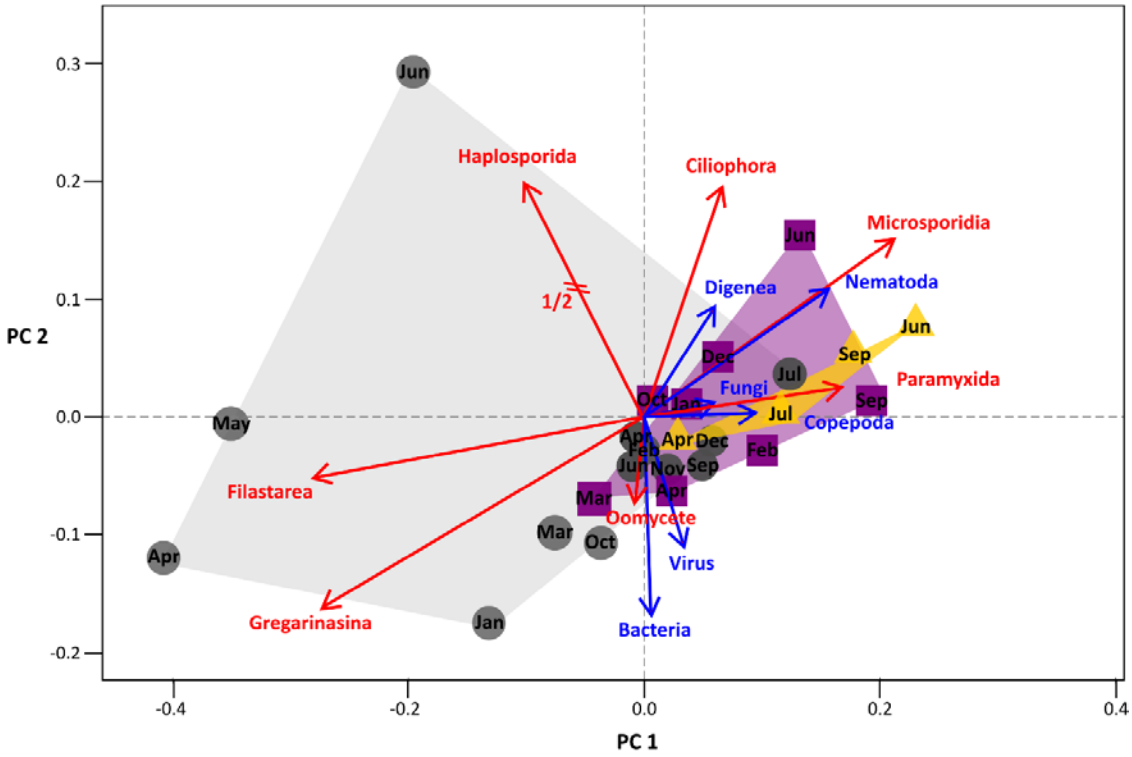


Figure 13: Principal Component Analysis (PCA) with parasites (arrows) as variables (protists in red; non-protist in blue). Observations correspond to the incidence of the different parasites (protist and non-protist) on each amphipod host (*Echinogammarus* sp.: grey circle, *Gammarus* sp.: purple square, and *Orchestia* sp.: yellow triangle) withthe month of the sampling specified inside. The coloured areas comprise the observations for each of the three amphipod hosts. The variation explained by the two main components (PC1 and PC2) was 75%.

3.5 Co-occurrence analysis of parasitic infections in individual amphipods

Certain parasites and symbionts associated to the amphipod community in Newton's cove appeared to have marked seasonal prevalences (Fig. 12, 13). Infections caused by some of these parasitic clades, like Haplosporidia, Filasterea, Paramyxida, or Microsporidia were virtually restricted to specific moments of the year in *Echinogammarus* sp. hosts but persisted throughout the year in amphipod genera *Gammarus* and/or *Orchestia*. Either way (short-lived or uninterrupted), infections frequently overlapped between them and with the rest of protist and non-protist organisms associated to that specific amphipod population. A force-directed Fruchtermann-Reingold-based network was constructed to investigate the co-occurrence of infections in individual organisms, and then combined with a Pearson's chi-squared test for independence correlation (Fig. 14). In all three amphipod species analysed, ciliates (associated to almost every individual amphipod studied) were in the centre of the network. The rest of the nodes, representing the other parasites/symbionts, were attracted to the centre and between each other depending on the number of individual amphipods in which they co-occurred, while being repelled if unconnected. The proximity between Microsporidia and Paramyxida nodes

suggested that these two infections co-occurred in individuals belonging to all amphipod species. A Pearson's chi-squared test confirmed that infections did not occur independently, especially in *Echinogammarus* sp. (χ^2 = 61.76, df = 1, p-value = $3.87e^{-15}$), but also in *Gammarus* sp. (χ^2 = 12.31, df = 1, p-value = 0.00045), and *Orchestia* sp. (χ^2 = 8.71, df = 1, p-value = 0.0031). Similarly, numerous *Echinogammarus* sp. individuals appeared to be co-infected by haplosporidian and filasterean parasites, a connection observed to be non-independent (χ^2 = 13.26, df = 1, p-value = 0.00027).

3.6 Relating temporal variability of the amphipod-associated pathobiome analysed in Newton's Cove to other locations in south-west UK

Equivalent histopathological analyses to the one conducted in Newton's Cove to investigate the parasitic/symbiotic community associated with *Echinogammarus* sp., *Orchestia* sp. and *Gammarus* sp. populations were conducted in other three coastal locations in southwest England (Fig.1). Ciliates and gregarines remained the most abundant protists associated with amphipods (Fig. 15). The incidence of ciliates appeared to be consistent in the four sampling locations (Newton's Cove, Dart estuary, Tamar estuary, and Camel estuary), with values ranging between 70% and 100%, slightly higher during spring (p-value< 0.05, Kruskal-Wallis test).The fluctuating prevalence of gregarines in amphipods sampled from Newton's Cove(Fig. 12), was consistent in the other locations as well. However, prevalence appeared to be significantly higher in estuaries than in Newton's Cove (p-value < 0.05, Student's t-test), with values above 50% in several estuarine locations for most of the year (Fig. 15). While microsporidian and paramyxid infections peaked during late summer in the *Echinogammarus* sp. population of Newton's Cove (Fig. 12), both parasites appeared to occur throughout the year in Dart, Tamar, and Camel estuaries. In fact, infection prevalence by these two protist parasites was significantly higher (p < 0.05, Student's t-test) in estuaries (*Dictyocoela* sp. = 20.2%; *Paramarteilia* sp. = 18.1%) than in Newton's Cove (*Dictyocoela* sp. = 11.9%; *Paramarteilia* sp. = 6.2%), and with a bi-modal distribution; with a second peak of infection during winter in addition to the one observed in summer (Fig. 15A). All three estuaries presented similar prevalences of microsporidian and paramyxid infections throughout the year. Furthermore, the incidence of both parasites appeared to be almost identical, something particularly evident in *Echinogammarus* sp. populations from Dart and Tamar estuaries (Fig. 15A). Seasonality of microsporidian and paramyxid infections in *Orchestia* sp. and *Gammarus* sp. in Newton's Cove (Fig. 12) were not as marked as in *Echinogammarus* sp. Microsporidian infections occurred throughout the year while paramyxids appeared to be especially abundant during summer and autumn, a pattern maintained in the estuaries (Fig. 15).

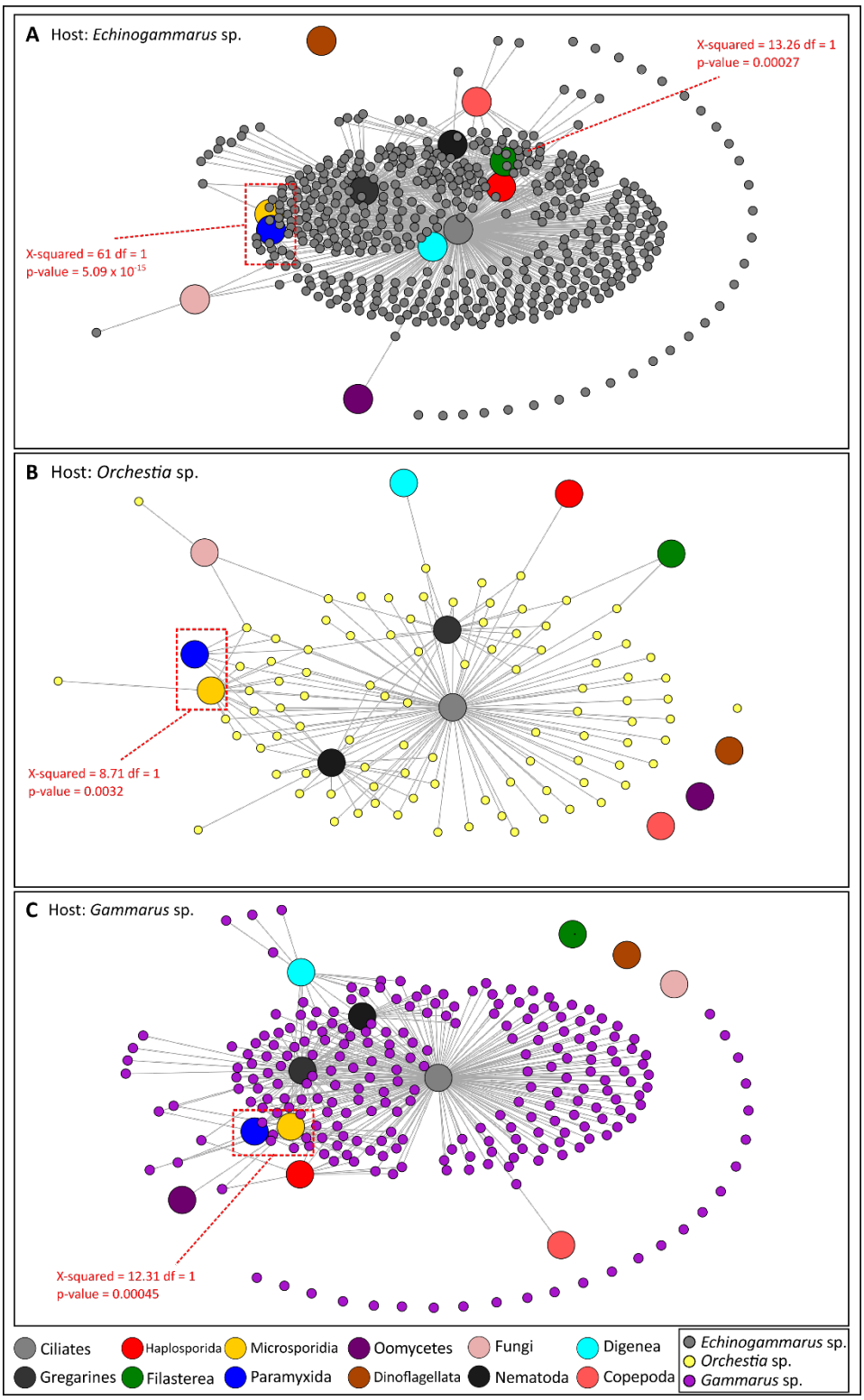


Figure 14: Force-directed association network based on the Fruchtermann-Reingold algorithm indicating which parasites (large spheres) co-occur within every single specimen examined (small spheres), for each amphipod genus (*Echinogammarus*, *Gammarus*, and *Orchestia*). The results of a Pearson's Chy Square Test appear indicated in those associations in which the network shows high level of co-occurrence. Notice how that parasites that were not observed in a certain amphipod genus and host specimens without any apparent infection or connection to a parasite/symbiont appeared in the periphery of the network,

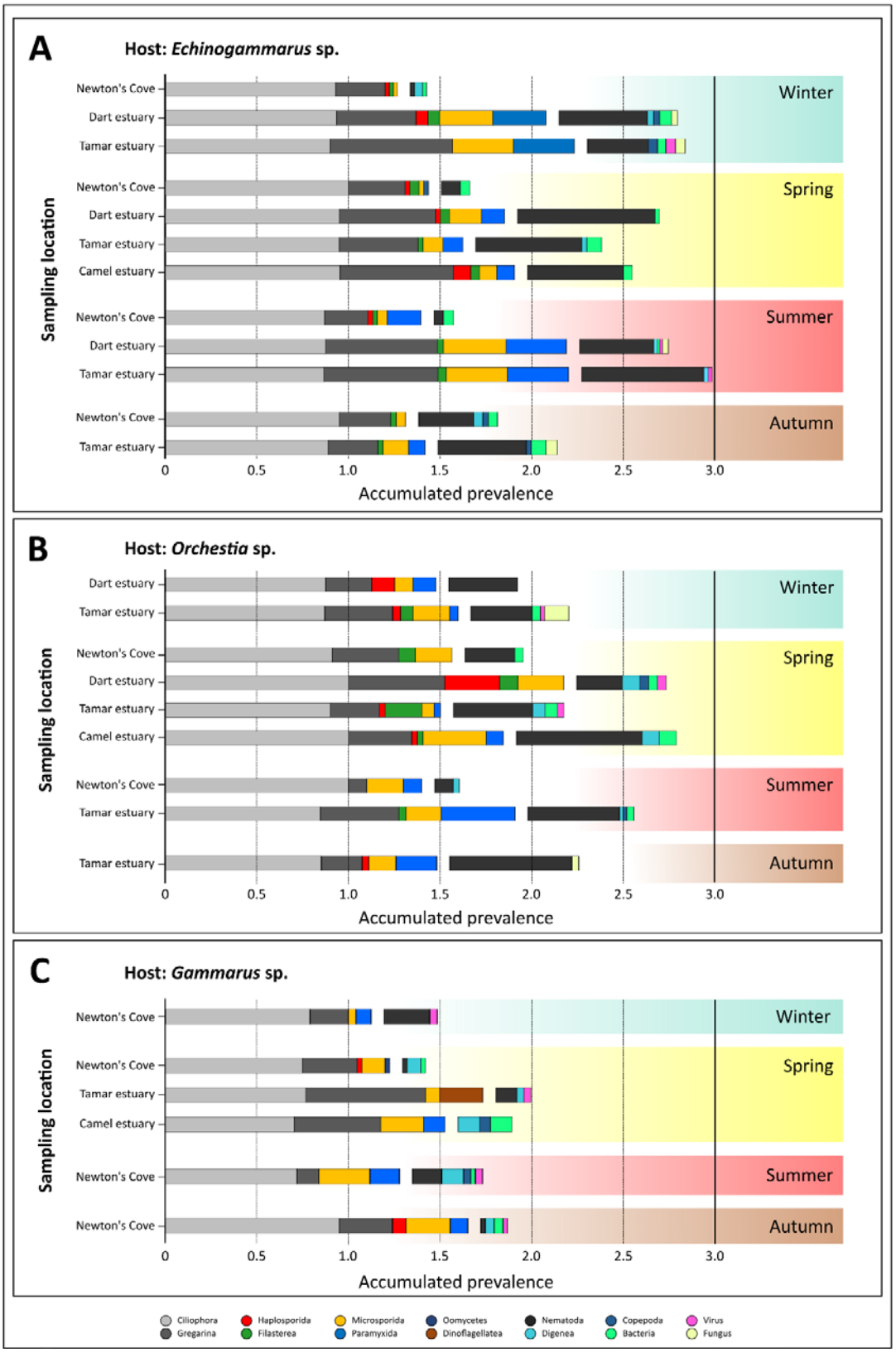


Figure 15: Bar chart showing the accumulated prevalence of protists and non-protist parasites (separated by a space) associated to *Echinogammarus* sp., *Orchestia* sp., and *Gammarus* sp. amphipod populations collected in Newton's Cove, Tamar estuary, Dart estuary, and Camel estuary during Winter, Spring, Summer, and Autumn.

During the short-lived infection peaks with haplosporidian and filasterean parasites up to two thirds of the *Echinogammarus* sp. population in Newton's Cove resulted affected (Fig. 12). Filasterean infections were also detected in *Echinogammarus* sp. collected from the other three estuaries, but the incidence was considerably lower (Fig. 15). In turn, haplosporidian-infected *Echinogammarus* sp. individuals were not detected in Tamar estuary, and the highest prevalence (10%) was observed in Camel estuary during spring. In contrast, no haplosporidian infections were observed in *Orchestia* sp. sampled in Newton's cove, but they occurred throughout the year in estuarine water, being highest in Dart estuary during spring. Filasterean microcells were observed throughout the year in *Echinogammarus* sp. and *Orchestia* sp., although virtually restricted to gonadal tissues and with low prevalence. Infection reached its highest (11%) in Tamar-sampled *Orchestia* sp. individuals, also during spring as it occurred in Newton's Cove sampled *Echinogammarus* sp. While differing in prevalence values, most protist parasites observed in Newton's Cove occurred in the other locations, with the exception of dinoflagellates. The haemolymph-congesting syndinian was only observed in *Gammarus* sp. collected in Tamar estuary during spring with a relatively high prevalence (30%) (Fig. 15C).

Non-protists parasites, including metazoans, fungi, bacteria, and viruses were also included in the analysis (Fig. 15). Nematodes were the most abundant metazoan parasites associated to amphipods, especially in estuaries (prevalences varying between 40% and 80%). They were significantly more prevalent in amphipod genera collected in the upper intertidal (*Echinogammarus* sp. and *Orchestia* sp.) than in *Gammarus* sp. collected behinds rocks (purple area) in the lower intertidal horizon. Fungal parasites were observed in *Orchestia* sp. and *Echinogammarus* sp. during autumn and winter (Fig. 15A, 15B). Bacteria and virus occurred in all amphipod species and locations, but incidences were generally low, and infections did not appear to follow a seasonal pattern (Fig. 15).

The accumulated prevalence of all parasites observed in every location and season (Fig. 16A) showed that the two amphipod genera sampled in the upper part of the intertidal (*Echinogammarus* sp., *Orchestia* sp.) were significantly (p-value < 0.05, One-way Anova; $p < 0.05$, Tukey HSD test) more parasitized than *Gammarus* sp., which inhabited the lower part of the intertidal zone. However, the difference was not significant (p-value> 0.05, One-way Anova) when only protist parasites were analysed. An equivalent analysis comparing the accumulated prevalence of parasites in the different estuaries (Fig. 16B) outlined significant differences for all parasites (p-value < 0.05, One-way Anova; p-value < 0.05, Tukey HSD test) and protists parasites alone (p-value < 0.05, Kruskal-Wallis test; $p < 0.05$, pairwise Wilcox test). Amphipods collected in Dart and Tamar estuaries were the most parasitized, followed by Camel and finally the coastal location of Newton's Cove. Finally, no significant differences (p-value > 0.05, One-

way Anova) were observed between seasons based on accumulated total prevalence of parasites (Fig. 16C).

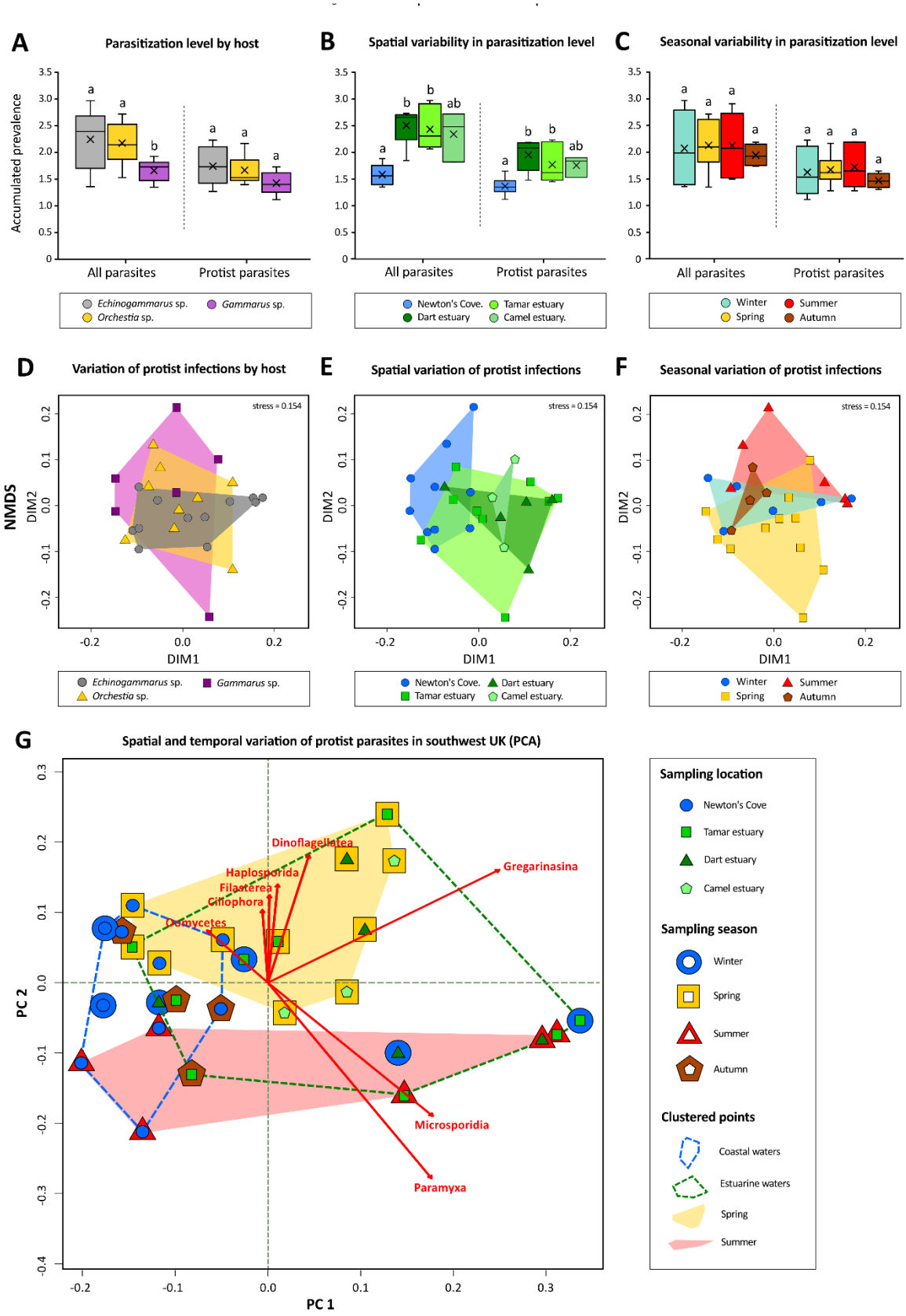


Figure 16: (Preceding page). Statistical and multivariate analysis illustrating the variability observed in parasitisation level for different amphipod species, locations, and seasons. **(A, B, C)** Boxplots illustrating the average and standard deviation in the accumulated prevalence of protists and non-protist parasites infecting amphipod genera *Echinogammarus*, *Orchestia*, and *Gammarus* **(A),** separated by location **(B)** and season **(C).** Letters "a" and "b" over the boxes are used to indicate significant differences between groups (p-value > 0.05, One-way Anova Test). **(D, E, F)** Non-metric multidimensional scaling (NMDS) illustrating variability (Bray-Curtis dissimilarity) in the occurrence and prevalence of parasites infecting amphipods grouped by host **(D),** sampling location **(E),** and season **(F). (G)** Principal component analysis (PCA) of the occurrence and prevalence of protist parasites (variables) infecting amphipods collected in different locations and seasons. The legend shows the relationship between shapes and areas.

Dissimilarities (Bray-Curtis) in the occurrence of the different protist parasites affecting amphipod species were represented graphically by NMDS (Fig. 16D). None of the three amphipod species could be distinguished from the others based on the matrix constructed combining occurrence and prevalence. However, the pathogen community associated with *Echinogammarus* sp. populations appeared to vary less than the one linked to *Gammarus* sp. An equivalent NMDS analysis conducted splitting those observations by sampling location (Fig. 16E) showed differences between the pathobiome present in coastal amphipod populations (Newton's Cove) and estuarine ones (Tamar, Dart, and Camel). While no significant differences were observed in the accumulated prevalence of protist parasites during different times of the year (Fig. 16C), the NMDS (Fig. 16F) showed that the composition and abundance varied greatly depending on the season, especially between spring and summer. A PCA(Fig. 16G) of the same observations used in (Figs. 16D, 16E, and 16F), outlined the amphipod-associated protist lineages contributing most to the spatial and temporal variability observed. Gregarines together with microsporidian and paramyxid parasites correlated positively (0.248, 0.182, and 0.177, respectively) with the main axis (PC1), and oomycetes negatively (-0.072). The rest of the endpoints, representing lineages Dinoflagellatea, Haplosporida, Filasterea, and Ciliophora correlated positively (0.186, 0.146, 0.132, and 0.104) respectively, with the second component (PC2). In consequence, differences between coastal and estuarine parasitic communities in the amphipod population were largely attributable to increased levels of microsporidian and paramyxid infections, and gut-associated gregarines in estuaries. In turn, seasonal differences were predominantly driven by short-lived filasterean, haplosporidian and syndinian infections occurring during spring, and more prevalent microsporidian and paramyxid infections during summer.

4. Discussion

4.1 General ecology of Newton's Cove

The structure of benthic communities in rocky coasts is invariably patchy in space and time (Creese & Kingsford 1998, Miller & Ambrose 2000). The continuous and heterogeneous mosaic of environments shaped by rocks, pools, ridges, and fissures allows for great diversity of species

and assemblages (Juanes et al. 2008). Distribution, population dynamics, and interactions between organisms change in response to a combination of physical, chemical, and biological factors, all profoundly influenced by a gradient throughout the vertical profile (Steyaert et al. 2003, Wilms et al 2006). Based on the above, the area from Newton's Cove selected to study the macrobenthic community present in the site was delimited to include a ridge, numerous ponds and rocks, and a steady inclination (between 3% and 4%). In total, 62 different invertebrate species were identified in a total sampled area of 2.125 m^2, a figure in the high range but consistent with equivalent studies conducted in the intertidal zone of other coastal locations in the British Isles and the North Atlantic (Rodrigues et al. 2006, Chapman & Underwood 2009, Høgslund et al. 2014). The SAC (Species Accumulation Curve) is not asymptotic yet and current data do not confirm that it follows the typical Michaelis-Menten equation type (Tjørve 2003) or a more logarithmic progression observed in ecosystems with numerous rare species (Dove & Cribb 2006). Assuming an asymptotic distribution, 15 would be the limit of invertebrate speciesthat remain undiscovered in the studied area, as 77% of the total species (n=48) had been identified in half of the quadrants examined. However, the heterogeneous and patchy environments created in rocky coasts often support a myriad of rare species that result in non-asymptotic SACs (Reichert et al. 2010), in which the actual number of species might be significantly higher. A modest increase in the number of quadrants sampled in the lower intertidal, especially in the area forming the ridge in Newton's Cove ("Brown area" in Fig. 1) would assist in a better interpretation of the function shaping the curve, and a better estimation of species richness (Chapman & Underwood 2009).

An adequate assessment of species richness is important to understand the composition and functioning of the community (Petchey & Gaston 2002), but a deeper analysis of extrapolation methods, estimator selection, or sampling effort optimization are beyond the scope of this work. The main objective of this ecological analysis in Newton's Cove was to identify abundant macrobenthic species, and to examine their role as hosts or vectors for significant protists parasites. Rare or occasional species might act as hosts/reservoirs of parasites as well, although incidence is generally considered to be constrained (not exclusively) by host population density (Arneberg 2001, Choisy et al. 2003, Stien et al. 2010). Besides, screening of rare species has in principle a higher impact on the ecosystem, and sampling effort increases significantly, especially in the case of frequent temporal analyses as the one intended. A second objective of this ecological analysis is to gain some direct insights into the invertebrate community present in the cove, which should assist drawing hypothesis and explaining the occurrence and aetiology of the pathogens documented in the location.

According to the rank-abundance analyses, highly abundant species in Newton's cove occur in the upper part of the intertidal zone. In contrast, the number of individuals quantified for species inhabiting the lower intertidal is substantially lower. This layout with higher species richness and diversity (increased evenness) in the lower intertidal zone is typical of rocky coastal environments (Scrosati & Heaven 2007) and has been associated with less stressful conditions (Tomanek & Helmuth 2002). While the upper zone of the intertidal is characterized by fewer but very abundant species (*Echinogammarus* sp., *Capitella* sp. *Procerodes* sp., and harpacticoid copepods), almost half of the species in the lower intertidal were only observed once or twice, a result shared by studies involving much greater sampling effort (Borja et al. 2010). The NMDS analysis computing dissimilarity between the different zones of the intertidal based on species occurrence and abundance, indicates a clear division between the upper ("Yellow", "Grey" and "Green" areas) and the lower intertidal zones ("Purple" and "Brown" areas). This outcome suggests that the time-consuming predefinition of the upper intertidal in different habitats based on substrate and algal coverage was not essential. However, the division between "brown" and "purple" areas in the NMDS outlines the significant impact in the community structure caused by the ridge dividing Newton's Cove lower intertidal in two (Fig. 1). This finding advises for stratified random samplings in locations with equivalent natural formations, expanding the circumstances and supporting its appliance in heterogeneous intertidal zones (Miller & Ambrose 2000, Schoeman et al. 2003, Van De Werfhorst & Pearse 2007). Besides, such stratified random samplings are suitable to define the transition area between upper and lower intertidal zones (Wallenstein & Neto 2006) as well, which in Newton's Cove corresponds to the "green" area (first algal assemblages constituted by genera *Blindingia*, *Ectocarpus*, *Ulothrix*, and *Ulva* attached to naked rock).

Crustaceans (amphipods, copepods, isopods), annelids (polychaetes), platyhelminths (turbellarians), and molluscs (shellfish, snails) dominate the invertebrate community present in Newton's Cove. These clades are pivotal in the archetypal community structure observed in benthic assemblages throughout the British Isles (Crisp & Southward 1958, Southward et al. 1995, Callaway et al. 2002) and rocky coasts in the North Atlantic (Raffaelli & Hawkins 2012). Possibly, the main divergence with some of these studies is constituted by the abundance of clade Echinodermata, which although present in Newton's Cove (sea stars, brittle stars) was not predominant. Besides, in North-western Atlantic rocky shores, from the north of the British Isles down to Portugal, the low intertidal is dominated by a turf of red algae, in which limpets and barnacles prevail (Lewis, 1964, Saldanha, 1974). Both groups have been shown to be abundant in the "brown area" defined in Newton's Cove, but none exerts clear dominance.

Multivariate methods of classification and ordination have been extensively used to compare communities based on identity (of the component species) and relative abundance / biomass (Warwick & Clarke 1991, Kleyer et al. 2012, Warton et al. 2012). The occurrence, distribution and relative abundance of the benthic invertebrate species inhabiting the type-location in Weymouth, has been investigated by PCA, using species as variables, as in equivalent studies analysing coastal communities (Davíd et al. 2005, Alfaro 2006). The non-rarefied analysis substantiates the importance of *Echinogammarus* sp. in the uppermost intertidal, cohabiting with highly abundant genera *Capitella* and *Procerodes*, although the later dominate in the intertidal belt 2-3 meters below (grey area). All three taxa have been shown to surpass the 1,000 individuals per square meter sampled, but their abundance can be several orders of magnitude higher in coastal ecosystems throughout the English Channel and the North Atlantic (Marques & Nogueira 1991, Boaden 1995, Ramskov & Forbes 2008). In conjunction with harpacticoid copepods, they represent a common food source for bigger invertebrates and vertebrates including crabs, lobsters, fish, and birds (Reise 1979, Stoner & Buchanan 1990, Trowbridge et al. 2018, Giari et al. 2020), offering a possible route of direct transmission already documented for metazoan parasites (Bauer et al. 2005, Keeney et al. 2007). The rarefied analysis neglects the representation of abundances in favour of a clearer portrayal of species distribution, outlining the occurrence in the upper intertidal of organisms such as *Anurida maritima*, *Orchestia* sp., or *Littorina saxatilis,* all of them highly resistant taxa to desiccation (Zinkler et al. 1999, Henzler & Ingólfsson 2008).

Besides, the heatmap and PCA indicate that the larvae and juveniles of these abundant habitat-specific organisms are more common in lower zones of the intertidal than their adult counterparts are. A result that is consistent with previous investigations on the distribution of amphipods and other little invertebrates (Craig 1973, Gosselin & Qian 1997) but opposed to the distribution of certain clades, such as crabs (Hunter & Naylor 1993). The target habitats for the different life-stages of invertebrates inhabiting coastal ecosystems appears to be a trade-off between factors including resistance to desiccation (Strachan et al. 2015), decreased predation and/or intra-specific cannibalism (Hochberg 1999), and food availability (Taghon 1982). However, the observation of juveniles reaching higher zones of the intertidal as they grow supports the description of horizontal benthic migration of amphipods and other invertebrates already described by Williams (1995), in conjunction to the protist parasites associated to them, as hypothesised by Curtis (1990).

Contrasting with the dominance of detritivorous feeding strategies among species inhabiting the upper intertidal, the opening of the first algal assemblages promotes the

expansion of herbivores and deposit feeders such as *Melita* sp. *Notomastus* sp., *Littorina* spp. or halacarid mites (Sommer 1999, Guidone et al. 2015). All well documented inhabitants of the intertidal zone in the British Isles (Goss-Custard et al. 1991). The shelter offered by this first algal assemblages consents the presence of their natural predators, including crabs *Cancer pagurus* and *Carcinus maenas*, which are highly motile invertebrates in coastal ecosystems (Robinson et al. 2011) that feed on *Echinogammarus* sp., *Capitella* sp., *Procerodes* sp., and harpacticoid copepods as well (Mendonça et al. 2009). Furthermore, the crab population sampled in Newton's Cove has been shown to be affected by numerous protist parasites including microsporidians, haplosporidians, paramyxids, or dinoflagellates (Stentiford et al. 2003, Stentiford et al. 2007, Feist et al. 2009, Stentiford et al. 2013, Ward et al. 2016).

Increasing diversity concurs with a decrease in the dominance of species inhabiting the lower intertidal, a community distribution widely documented in European rocky shores (Boaventura et al. 2002). Isopods (*Jaera ischiosetosa*, *Dynamene bidentata*), gastropods (*Littorina* spp., *Gibbula* sp. *Epitonium* sp.), and annelids (*Pomatoceros triqueter*., *Spio* sp., *Spirorbis* sp.) are frequent in ponds behind rocks and algae in the lower intertidal, a distribution well documented by historical works such as the one conducted by Moore (1939), in the nearby coastal location of Plymouth. However, these abundant lower-intertidal invertebrates do not form the compact clusters observed for species inhabiting the upper intertidal, possibly due to milder abiotic conditions and sites to hide (Scrosati & Heaven 2007). Predominantly herbivorous amphipod genera *Gammarus*, *Hyale*, *Caprella*, *Ampithoe*, and *Abludomelita* are frequently observed in the lower levels of the intertidal, hidden inside rock-holes and amongst algal fronds, reason why they lack the burrowing and jumping behaviours cahracterizing amphipods inhabiting upper intertidal zone (Hurley 1968, Aikins & Kikuchi 2001, Rossano 2008). The invertebrate community in the most exposed zones of the lower intertidal zone in Newton's Cove is dominated by gastropods and filter feeders, including the crustacean *Chthamalus* sp. and the molluscans *Patella* sp., and *Mytilus edulis*. The later are usually observed in greater abundances in comparable rocky shores in the British Isles (Connor et al. 2004). Partially protected by Portland's Harbour from the prevailing western winds, this area of Newton's Cove is conceivably determined by a particularly mutable wave regime depending on the prevailing current. While larvae of barnacles and limpets tend to settle in more wave-beaten habitats (Denny 2014), reason why they appeared in the most obvious ridges; *Mytilus* spp., prefer more protected areas (Steffani & Branch 2003), explaining their occurrence almost exclusively in crevices formed in Newton's Cove lowest intertidal zone.

In summary, some of the most abundant invertebrate species in Newton's Cove and comparable rocky shores in the British Isles are *Echinogammarus* sp. amphipods, *Capitella* sp. polychaetes, *Procerodes* sp. platyhelminths, and harpacticoid copepods (Warren 1976, Bodin & Leguellec 1992, Egilsdottir et al. 2009). All four lineages, which share the same habitat in the upper intertidal zone, have been shown be solidly connected between them and to larger predators in the food-web, including crabs, lobsters, fish, and birds (Boaden 1995, Mendonça et al. 2009, Alexander et al. 2012). In turn, some of these predators are extensively affected by a wide range of protist parasites in this area of the English Channel (Stentiford et al. 2003, Stentiford et al. 2007, Feist et al. 2009, Stentiford et al. 2013, Ward et al. 2016). Despite the documented trophic link, the pathogen community associated with genera *Echinogammarus*, *Capitella*, *Procerodes*, and harpacticoid copepods remains largely unknown. Consequently, these four lineages appear as sensible candidates for a full histopathological screening as putative reservoirs of micro-eukaryotic pathogens.

4.2 Histology and pathogens of common invertebrate species in Newton's Cove

The ecological importance of amphipods, copepods, polychaetes and platyhelminths for coastal communities has been extensively demonstrated (Martens & Schockaert 1986, Duffy & Hay 2000, Scaps 2002, Turner 2004, Beaugrand & Kirby 2010, Michel et al. 2015). Feeding on detritus, polychaetes play an indispensable role recycling nutrients while incidentally oxygenating the sediments (Giangrande et al. 2005, Ekeroth et al. 2016). Copepods represent a pivotal link for the community in the water column, where their vertical migration operates as a biological carbon pump (Sander et al. 2014). Diverse and abundant, amphipods are key species in many marine and freshwater ecosystems, functioning as predators, grazers, scavengers, detritivores, or even parasites (Macneil et al. 2011, Padovani et al. 2012, Riascos et al. 2015). However, most species in these clades remain insufficiently characterized histopathologically and/or metagenomically (if at all), and in consequence, the community of parasites and symbionts associated to them is largely unknown (Peoples et al. 2012, Dunlap et al. 2013, Bojko et al. 2019)

Several *Capitella* spp. including *C. Capitata* have been morphologically described by stereo-microscopy (Warren 1976) and scanning electron microscopy (Blake 2009), but our results represent the first histological description of organs and tissues for this species and genus; with the exception of the gonads, which were recently described in *C. biota* (Sampieri et al. 2020). Additionally, our findings suggest that this abundant and cosmopolitan polychaete genus (Tomioka et al. 2016), widely used in toxicological assays (Bach et al. 2005, Dai et al. 2015) and cell regeneration research (de Jong et al. 2016) might be a suitable candidate for

histopathological screenings as well. Their small size (1-2 cm) allows a good fixation of full-body individuals in batches, and their reduced prostomium and smooth setae make it easier to section than larger and more frequently screened polychaetes such as *Nereis* sp., or *Nephthys* sp. (Lynch et al. 2007). The structure of all major organs and tissues including gut, gonads, tegument, muscle, and connective is well retained, easing the histological analysis. The histopathological analysis of *C. Capitata* individuals sampled from May to November in Newton's cove has shown that ciliates and gregarines are the most frequently observed protists associated to the polychaete, especially during late summer and autumn. The morphology of the gregarine resembled that of *Ancora sagitatta*, a long-known parasite infecting the gut of *C. Capitata* (Schrével & Philippe 1993, Simdyanov et al. 2017). In contrast, ciliates have been observed to be either part of the diet, or epibionts in *C. capitata* (Alongi 1985); although parasitic ciliates have also been described in Family Capitellidae (Mikac et al. 2020). The parasitic microsporidian observed inside the intestine cells of *C. Capitata* may well be *Amphiamblys capitellides*, a hyper-parasite of *Ancora sagittata* (Larsson & Køie 2006). However, metchnikovellid microsporidians appear to be highly diverse and common hyper-parasites of polychaetes as well (Sokolova et al. 2014, Rotari et al. 2015); consequently, either molecular or ultrastructural analyses will be necessary to confirm the species.

The remarkable ability of planarians to avoid infection (Peiris et al. 2014) and regenerate after amputation (Reddien & Alvarado 2004) are being increasingly investigated at molecular and histological level (Cebrià 2007, Abnave et al. 2014). However, and despite its abundance and widespread distribution in the North Atlantic (Barnes 1994), *Procerodes* sp. has not been histologically characterized until now. The irregular shrinkage of the body after fixation, and the intricate disposition of the organs in a connective-like matrix; together with an open digestive system and disseminated food particles, are major setbacks for the histopathological analysis of this and possibly other planarians (Kolasa & Tyler 2010). As for *C. capitata*, ciliates and gregarines are the main protists associated with *Procerodes* sp., but their incidences are lower than in the polychaete, with maximums occurring during early summer rather than in late summer/autumn. In the turbellarian, ciliates do not occur inside the gut lumen, but in the interstitial sinuses surrounding the pharynx, and several genera such as *Steinella* or *Tetrahymena* have been identified as parasites of *Procerodes* sp. and other planarians (Boaden 1995, Rataj & Vďačný 2020). In turn, there is no record of parasitic/endosymbiotic gregarines in *Procerodes* sp., although few species have been observed to parasitize other planarian genera (Harrath et al. 2013). The frequent occurrence of bacteria in the gut and connective tissues of *Procerodes* sp. was unexpected, as the immune system of planarians is known to be highly efficient against

bacterial infections (Vila-Farré 2018). Therefore, they will be most likely symbiotic, as no evident host response was observed, although parasitic associations have been documented in other genera (Kangale et al. 2020).

Copepods are estimated to represent over half of the total metazoan biomass in marine ecosystems (Turner 2004), making them a pivotal link between primary producers and secondary consumers, from slightly bigger invertebrates to marine mammals (Roman & McCarthy 2010). Accordingly, copepods have been extensively regarded as possible reservoirs/intermediate hosts for numerous parasites (Støttrup 2000), but the connection is rarely demonstrated (Jones et al. 2012). Although molecular-based methods often suggest possible associations (Burreson 2008), infection is rarely subtantiated histologically, probably due to complex host identification, small size, and challenging methodology. However, a double embedding technique using agar (Feist & Bucke 1983) allows straight forward fixation and histological processing. While the general structure of tissues and organs is perfectly maintained, the reduced cell and nuclear size, in the range of many micro-eukaryotic parasites, holds back the detection of microcell pathogens. Few authors (Audemard et al. 2002, Arzul et al. 2014) have overcome this challenge by combining molecular and visual techniques such as in-situ hybridization (ISH) for specific pathogens, but the technique is neither straightforward nor apt for wide-ranging pathogen screenings. The histopathological analysis of the harpacticoid population present in Newton's Cove has shown copepods belonging to Ameiridae Family to be considerably less parasitized than polychaete, turbellarian and amphipod hosts; only ciliates, gregarines and bacteria have been identified. However, direct comparison was beyond the scope of this work and delicate, as prevalence has not been randomized per host tissue area scrutinized. In line with findings by Hockin (1984), who investigated the parasites/symbionts occurring in the harpacticoid population from Aberdeenshire coasts (British Isles), Ciliophora dominates among the copepod-associated protist lineages. Genera *Lecanophyra*, *Thecacineta,* or *Cothurnia* are common epibionts, often with great host-specificity (Guo et al. 2012), which also represent and important food source for the copepod (Calbet & Saiz 2005). Nevertheless, several species, including *Vampyrophyra pelagica*, *Dendrosomides lucicutiae*, or *Uronema* sp. have been described as important parasites (Ho & Perkins 1985).In contrast, there are no gregarine species described from harpacticoid copepods, although genera such as *Monocystis*, *Thiriotia*, and *Cephaloidophora* are known to infect calanoid and cyclopoid copepods (Takahashi et al. 2008, Sano et al. 2016).

The histopathological pre-screening of the *Echinogammarus* sp. population from Newton's Cove identified several protist-based infections that were absent, or infrequent in the

other invertebrates examined. These preliminary results suggest that apart from being prominently abundant in Newton's Cove and coastal ecosystems throughout Europe (Alexander et al. 2012), *Echinogammarus* sp., and amphipods in general might represent an important reservoir for alveolate, opisthokont, and rhizarian protist parasites. Clades comprising numerous disease-causing lineages with a significant impact in fish, crustacean and bivalve aquaculture (Mendoza et al. 2002, Stentiford et al. 2013, Hartikainen et al. 2014, Ward et al. 2016, Stoeck et al. 2018, Rueckert et al. 2019). Consequently, a two-year longitudinal histopathological screening was conducted not only in *Echinogammarus* sp. but in other abundant amphipod species from Newton's Cove (section 4.3).

4.3 Histopathology, ultrastructure and molecular phylogeny of parasites infecting *Echinogammarus* sp. and other amphipod species

A key organism in macrobenthic communities throughout Europe and America (invasive in the later), *Echinogammarus* sp. is extensively used in bio-toxicity assays and environmental impact studies (Camargo & Alonso 2006, Egilsdottir et al. 2009, Bossus et al. 2014, Bruck & Ford 2018). Moreover, the amphipod is known to host a number of eukaryotic parasites, principally microcells from lineages constituting former Kingdom protista (Short et al. 2012, Guler et al. 2018, Bojko & Ovcharencko 2019). The complexity to identify by light microscopy these small (2-10 µm) organisms with reduced and often paraphyletic morphological traits (Vossbrinck & Debrunner-Vossbrinck 2005), has largely prevented the consideration of the host's pathogenic status into traditional ecological, toxicological, or environmental studies (Santoferrara et al. 2016). However, parasites are known to influence the outcome of such investigations (Marcogliese & Pietrock 2011). While PCR-based methods allow detection of specific parasites, and more recent metagenomic analyses allow characterizing the pathobiome of suitable hosts, microscopy remains indispensable to confirm infection and histopathology. Our results show that light microscopy allows identification of Class, Order, or even Family depending on the parasite clade. A process that substantially simplifies and speeds up the detection of pathogens to species/genus level, for which molecular techniques and/or ultrastructural analyses are usually required. A table summarizing traits with taxonomical value has been designed to identify protists clades associated to *Echinogammarus* sp. and other common amphipods from coastal ecosystems in the British Isles and Atlantic coasts of Europe (Jażdżewski 1980, Costa & Costa 1999, Henzler & Ingólfsson 2008). The guide is based on a combination of factors including size, morphology, tissue tropism and prevalence (Table 4).

As for polychaetes, turbellarians, and copepods, the alveolate clades Ciliophora and Gregarinasina are the most frequently observed protist lineages associated with *Echinogammarus* sp. hosts in Newton's Cove. The predominance of ciliates is unsurprising, as they represent one of the most common and abundant taxa in coastal ecosystems (Zhang et al. 2018), where they feed on bacteria occurring in water and sediments (Ichinotsuka et al. 2006). In order to increase their filtering activity, many ciliate species have evolved as epibionts of invertebrate hosts (Wahl & Mark 1999, Utz & Coats 2005, Bickel et al. 2012), an association that can be rather host-specific for some species and lineages (Fenchel 1965, Mikac et al. 2020). For instance, only within Peritrichia, one of the several ciliate subclasses, almost 300 species are known to be epibiotic in crustacean hosts (Fernandez-Leborans & Tato-Porto 2000), many of them, in amphipods (Clamp 1993, Gudmundsdóttir et al. 2018). In fact, most of the ciliate genera (*Vorticella*, *Cothurnia*, *Zoothamnium*, or *Rhabdostilla*) identified in the metagenomic analysis of *Echinogammarus* sp. and *Gammarus* sp. in Weymouth, belong to this clade. These molecular identifications correspond with microscopical observations, which indicate that most ciliates detected in amphipods are attached to the carapace, gills, pereopods, pleopods, and egg pouch.

However, commensalism is not the only symbiotic relationship occurring between ciliates and marine hosts; a substantial number of ciliates are parasitic, not only for invertebrates (Ohtsuka 2004) but for vertebrates as well (Dickerson & Clark 1996). While epibiotic, some ciliates such as *Conidophrys* spp. are regarded as parasitic, having an impact on numerous amphipod genera including *Gammarus*, *Melita*, and *Jassa* (Bojko et al. 2019). Besides, they are known to increase mortality at population level (Prokopowicz et al. 2010). Some of the ciliates associated to amphipods in Newton's cove appear to be able to penetrate inside the outer tegumental layers, especially in the gills, where the cuticle is thinner. A pathology that has already been documented for chonotrichid genera *Heliochona* and *Spirochona* infecting *Gammarus* sp. and *Hyale* sp. from North America and Eastern Europe (Dovgal & Grigorovich 2001, Fernandez-Leborans et al. 2016). The metagenomic analyses of *Echinogammarus* sp. and *Gammarus* sp. in Newton's Cove have detected the related chonotrichid genus *Isochona* whose ecology remains undetailed. Rarely, ciliates invading the gills and somatic tissues of genera *Echinogammarus* and *Orchestia* were observed, a highly unusual infection already described for *Gammarus roeselii* and *Themisto libellula* (Chantangsi et al. 2013, Bojko et al. 2017). The metagenomic detection of genera such as *Philaster* and *Gimnodinoides* in amphipods from Newton's cove, taxa known to cause infection in copepods, isopods, and corals (Cook & Chubb 1998, Sweet & Bythell 2015), substantiate the need for detailed histological and molecular screenings of amphipods as reservoirs for significant ciliate parasites.

In contrast to the predominant ectocommensalism/ectoparasitism of Ciliophora, organisms in clade Gregarinasina are exclusively obligatory endosymbionts or endoparasites of invertebrates (Leander et al. 2008). Over 1650 species of eugregarines (the largest of the three traditional categories) have been described and many more are believed to exist (Adl et al. 2007). This lineage is further divided into two clades, 'Septatorina' and 'Aseptatorina', based on the presence of a septum dividing the trophozoites (the feeding form) in two. The septate lineage, to which all gregarines observed in amphipods from Newton's Cove correspond (microscopical analysis), includes most existing genera and species, known to parasitize predominantly insects and crustaceans (Rueckert et al. 2019). Although historical reviews based on morphological traits hint a greater number (Desportes 1972, Levin 1977), only 10 gregarine species have been described from infected amphipods using microscopical and molecular methods (Bojko et al. 2019). The phylogenetic analysis of these amphipod-infecting genera (*Cephaloidophora*, *Heliospora*, *Ganymedes*, *Thiriotia*, *Uradiopora*, *Rotundula* and *Frenzelina*) placed all of them in clade Cephaloidophoroidea, a "crustacean gregarine clade" (Rueckert et al. 2011, Simdyanov et al. 2015, Diakin et al. 2019), which includes all known gregarines from crustacean hosts. At least 48 different environmental OTUs (many of them isolated from the guts of krill and bivalve molluscs) were comprised within this clade, many of them closely related to sequences from amphipod-infecting gregarines. Similarity among sequences also permitted to argue that genera *Cephaloidophora* and *Heliospora* were less host-specific than anticipated, in comparison to highly host-specific eugregarines from other lineages (Lantová et al. 2010). This finding is supported by our metagenomic analysis, which shows that both *Gammarus* sp. and *Echinogammarus* sp. sampled in Newton's Cove, are infected by *Heliospora* sp. The genus is constituted by two species, *H. longissima* from *Gammarus pulex*, *Acanthogammarus godlevskii*, and *Eulimnogammrus* sp; and *H. caprellae* infecting *Caprella* spp. While the pathogeny of *Heliospora* sp. and other amphipod-infecting gregarines is poorly understood (Prokopowicz et al. 2010, Grunberg & Sukhdeo 2017, Bojko et al. 2019) the occurrence of gregarines in high numbers is known to affect host development, size, and reproductive capability (Marden & Cobb 2004, Gigliolli et al. 2016, Rueckert et al. 2019). Such could be the case of gregarines infecting genera *Echinogammarus*, *Orchestia*, and *Gammarus*; occasionally observed to host a myriad of individual trophozoites attached to the gut lumen. Additionally, intracellular stages (most likely sporozoites) have been seldom noticed inside intestinal cells, and even invading the connective tissues around the gut, eliciting an inflammatory host reaction. Equivalent pathologies have already been reported in polychaete and decapod-infecting gregarines (Lightner 1996, Landers & Leander 2005). In summary, whilst gregarine-associated pathologies in amphipods are not

frequent, amphipods represent a reservoir for species shown to be generalist endoparasites, in which the pathogenesis has only been superficially explored.

Part of the SAR supergroup as well (Simpson & Roger 2002, Burki et al. 2020), but substantially smaller than ciliates and gregarines, the rhizarian Class Ascetosporea Cavalier-Smith 2002 is constituted exclusively by obligatory parasites of invertebrates (Bass et al. 2019). The interest for this clade is quickly increasing, fuelled by phylogenetic analyses backing the incorporation of important orphan pathogen lineages and much greater diversity than anticipated (Cavalier-Smith & Chao 2002, Hartikainen et al. 2014, Ward et al. 2018, Bass et al. 2019). Five orders constitute Class Ascetosporea: Haplosporida Caullery & Mesnil*,* 1899; Paramyxida Chatton, 1911; Claustrosporida Larsson*,* 1987 (2003); Paradinida Cavalier-Smith, 2009; and Mykrocytida Hartikainen, Stentiford, Bateman, Berney, Feist, Longshaw, Okamura, Stone, Ward, Wood & Bass*,* 2014. Two of the orders (Haplosporida and Paramyxida) have been shown to frequently parasitize *Echinogammarus* sp., *Gammarus* sp., and *Orchestia* sp. populations inhabiting Newton's Cove.

Although the similar size of uninucleated cells does not allow to discriminate between the two clades, the oval shape and central nucleus of haplosporidian cells contrast with the spherical cells and peripheral nucleus of amphipod-infecting paramyxids. Morphological differentiation between the two ascetosporean lineages is considerably easier when dividing stages are present. While in amphipod-infecting haplosporidians a multinucleated plasmodium (without internal cleavages) encloses up to 20 identical nuclei (Messick & Faisal, 2014, Urrutia et al. 2019) in amphipod-infecting paramyxids "daughter cells" (membrane-bound secondary cells) arise by endogeny within a primary cell forming the characteristic "cell within cell" structure (Feist et al. 2009). The ornamented spores of haplosporidians are readily differentiated from the bi-cellular spores of paramyxids by the presence of a lid (Desportes 1984, Burreson & Ford 2004), but spore-formation was not observed by light microscopy or TEM in infected amphipods. Structural and molecular differences have shown that different haplosporidian species infect amphipod genera *Echinogammarus*, *Orchestia,* and *Gammarus*. In fact, these infections are caused by two novel haplosporidian species: *Haplosporidium echinogammari* n. sp. and *Haplosporidium orchestiae* n. sp. (discussed in Chapter 3; Urrutia et al. 2019).

In contrast, paramyxids infecting amphipods in Newton's Cove have identical 18S rDNA sequences (~ 420 bp), which have been shown to correspond to *Paramarteilia orchestiae.* This species was morphologically described by Ginsburger-vogel & Desportes (1979) but has gained attention more recently for its feminizing effect on the host (Pickup & Ironside 2018). Besides,

it has been hypothesized (Ward et al. 2016) that *P. orchestiae* might correspond, or be closely related to, crab-infecting *P. carcini*, whose DNA has not been sequenced yet. The parasite is known to infect edible crab *Cancer pagurus* (Feist et al. 2009) and remains together with *P. orchestiae* as the only crustacean-infecting paramyxid species. Our combined histopathological and phylogenetic analyses expand the known host range for *Paramarteilia orchestiae*, further supporting the view by Ward et al. (2016) that this species might be less host specific than conceived. A wider target host spectrum would have significant implications, as the same parasite has been detected in incubation water from *Cerastoderma edule* and *Mytilus edulis* (Ward et al. 2016). Life cycles including bivalve-infecting stages and intermediate crustacean hosts would not be extraordinary within lineage Paramyxida, as copepods have already been shown to be intermediate hosts for *Marteilia refringens*, the causative agent of Aber's disease (Audemard et al. 2002, Boyer et al. 2013). Infection of crabs, mussels, or cockles would escalate the significance of *P. orchestiae* as a pathogen of wild and farmed populations.

The prevalent occurrence of *P. orchestiae* in female eggs and gonads, as well as male testes is consistent with the predominant vertical transmission noticed by previous authors (Ward et al. 2016, Guler et al. 2018). Additionally, the parasite was frequently observed in ventral ganglia, eliciting inflammation, and causing damage to nervous tissues; a tissue tropism that was not documented in the original description by Ginsburger-Vogel & Desportes (1979). While nerve-associated damage has not been linked (neither studied) to increased predation in paramyxid-infected organisms (predominantly bivalves), it might significantly reduce the ability to escape from predators in crustacean hosts. A possibility already demonstrated for other protist lineages infecting nerves and ganglia in crustaceans, such as Amoebozoa or Dinoflagellatea (Messick-Walker & Zunt 2005). Actually, an evolutionary selected nerve targeting would back up the possibility of horizontal transmission in *Paramarteilia* sp. and other paramyxids as suggested by Guler et al. (2018), a clade in which vertical transmission is dominant (Berthe et al. 1998). Besides, reduced amphipod motility would further sustain the hypothesis of *P. orchestiae* infecting *C. maenas* (Ward et a. 2016)by showing a clear route of infection, as the crab is known to predate on amphipods (MacNeil et al. 2011).

Occasionally, filamentous fungal-like organisms belonging to clade Oomycetes (SAR, Stramenopiles) were noticed infecting the eggs of *Echinogammarus* sp. and *Gammarus* sp. which development into larvae within the female pouch was halted. Known as "water moulds", Oomycetes are generally thought of as saprotrophs or important plant pathogens (West & Beakes, 2014). However, they are also responsible for serious disease outbreaks and associated mortality events in farmed and wild animals (Gieseker et al. 2006). For instance, it is estimated

that around 10% of the world-wide salmon aquaculture is lost to *Saprolegnia* infections (Phillips et al. 2008). Besides, the clade is known to have an unusually large host-range, spanning from other protists, algae, plants, and fungi (Kamoun 2003) to invertebrate and vertebrate animals; including mammals and humans (Mendoza & Newton, 2005, Spies et al. 2016). Among crustaceans, Oomycetes have been shown to infect lobsters (Shields et al. 2011, Holt et al. 2018), crabs (Leafio 2002), shrimps (Nakamura et al. 1994), and crayfish (Filipova et al. 2013), often wiping-out entire populations (Shields 2012). However, there is limited evidence of amphipod-infecting Oomycetes, an association discovered not long ago by Kiziewicz & Nalepa (2008). To the date only five amphipod species: *Echinogammarus ischnus*, *Gammarus fasciatus*, *G. pulex*, *Diporeia* sp., and *Dikerogammarus haemobaphes* have been associated to Oomycetes (Bojko & Ovcharenko 2019), but infection has never been histopathologically demonstrated. While its occurrence in *D. haemobaphes* and *Gammarus pulex* was based on molecular methods (Sarowar et al. 2013, Bojko & Ovcharenco. 2019), Oomycetes affecting fresh-water amphipods *E. ischnus*, *G. fasciatus*, and *Diporeia* sp.(inhabiting the great lakes) were only visually detected in the carapace of death individuals (Kiziewicz & Nalepa 2008, Kestrup et al. 2011).

Phylogenetic analyses of these putative infections have shown that these amphipod-associated Oomycetes pertain to *Saprolegnia* sp., *Leptolegnia* sp., *Achyla* sp., and *Myzocitium* sp. For these parasitic genera, eggs represent one of the main target tissues in crustacean, amphibian, and fish hosts (Nakamura & Hatai 1995, Van West et al. 2006, Petrisko et al. 2008). Future work should determine the phylogenetic position and significance of oomycete-caused infections in amphipods from Newton´s Cove or other southern locations in the British Isles, as potential reservoirs of pathogens infecting commercially valuable crustacean species. The reason being that the lineage is constituted by generalist parasites known to infect crayfish and lobsters, in southwest England (James et al. 2017, Holt et al. 2018).

Two different lineages of microcells belonging to supergroup Opisthokonta are responsible for recurrent infections in amphipod populations inhabiting Newton's Cove. Microsporidia is a long-known phylum of obligatory protists parasites recently included in clade Holomycota/Nucletmycea to reflect its evolutionary proximity to Fungi (Brown et al. 2009, Liu et al. 2009, Corsaro et al. 2016). In contrast, phylogenetic/phylogenomic analyses have shown that the other parasitic opisthokonts infecting genera *Echinogammarus* and *Orchestia* belongs to Holozoa, a clade including animals and its unicellular relatives (Lang et al. 2002). This novel species, *Txikispora philomaios* n. sp., stands as the first confirmed parasite in Class Filasterea Cavalier-Smith, 2008, and type species for novel genus *Txikispora*, and Family Txikisporidae. The

histopathology, ultrastructure, genomic structure, biogeography, and ecology of the novel species are further discussed in Chapter 2 (Urrutia et al. 2021).

Over 1.500 species and200 genera have been described in Phylum Microsporidia, although the clade is considered to be much larger (Keeling 2009, Stentiford et al. 2019). All of them are obligatory parasites with a wide host range that extends from other protists to vertebrates, including humans (Vávra & Lukeš 2013). While insects and fish stand as the principal animal hosts, many invertebrate clades remain largely understudied (Stentiford et al. 2013). Besides, numerous microsporidian lineages are known to have complex life cycles, often including more than one intermediate host (Becnel et al. 2005, Grabner et al. 2015). Numerous, extraordinarily diverse, and intricately connected in the ecosystem, Microsporidia are regarded as emergent pathogens in the wild and the global food chains (Stentiford et al. 2016, Stentiford et al. 2019). From the 50 genera known to infect crustaceans (Stentiford et al. 2013), 13 have been shown to cause infection in amphipods, totalling 30 species and over 150 SSU and LSU rDNA sequences (Bojko & Ovcharenko 2019). While three of them: *Dictyocoela* sp., *Nosema* sp., and *Cucumispora* sp., account for half of the amphipod-infecting species, genera like *Fibrillanosema*, *Amblyospora*, *Thelohania*, *Pleistophora*, *Octosporea*, or *Anncaliia* are also significant. The phylogenetic analyses of the 18S rDNA genes have shown that microsporidian infections occurring in amphipods from Newton's Cove are caused by at least two different *Dictyocoela* species (not every microsporidian infection, n = 103, was molecularly analysed). *Echinogammarus* sp. is host to *D. duebenum*, which infects predominantly the amphipod's gonad, skeletal muscle, and integument. This species is widely distributed in Europe and Northern Asia (Bacela-Spychalska et al. 2018) affecting predominantly freshwater amphipods from genera *Gammarus*, *Eulimnogammarus*, and *Gmelinoides*. However, it has also been documented in several *Echinogammarus marinus* populations all around the British Isles (Guler et al. 2018). Populations in which the related species *D. berillonum* is also frequent (Terry et al. 2004). In turn, *Orchestia* sp. individuals from Newton's Cove appear to be infected by *D. cavimanum*, which affects predominantly connective tissues and hepatopancreas, eliciting a severe host reaction. This finding supports the apparent inclination of the species for talitrid amphipod hosts (Wilkinson et al. 2011). Species-level identification for genus *Dictycoela* is determinant, as vertically transmitted haplotypes tend to feminize infected hosts, while horizontally transmitted lines cause significant population mortalities (Wilkinson et al. 2011, Ironside & Alexander 2015, Pickup & Ironside 2018, Guler et al. 2018)

Although exclusively associated to *Gammarus* sp. sampled in the Camel estuary, one more alveolate protist (SAR supergroup) was identified causing infection in amphipods. The

parasite, a dinoflagellate, was observed congesting the host's haemal sinuses, the only tissue noticed to be affected. However, haemolymph-restricted infections coupled to an apparent absence of host reaction are characteristic among syndinean-caused diseases (Rowley et al. 2015). The ultrastructural analysis (TEM) showed the presence of uninucleated trophonts and bi-nucleated plasmodia comparable to those of *Hematodinium* sp. (Stentiford & Neil 2011) and its sister lineage, *Syndinium* sp. Chatton 1912 (Cachon & Cachon1987). The *Hematodinium* spp. represent a major cause of disease for over 30 decapod species (Stentiford & Shields 2005), especially crabs and lobsters; in which congestion of the haemal sinuses by parasitic trophonts causes high mortality and significant economic losses for crustacean aquaculture (Shields 1994). The genus is known to have a complex life cycle (Frischer et al. 2006), in which little invertebrates including amphipods have been hypothesized to act as reservoirs. Ever since Johnson (1986) detected histologically *Hematodinium*-like parasites infecting the haemolymph of 13 marine amphipod species in the north-eastern coasts of the USA, a number of studies have tried to demonstrate infection in amphipods by decapod-infecting lineages (Small 2004, Hamilton et al. 2009, Pagenkopp-Lohan et al. 2011, Small 2012). However, confirmation for such connection in the life cycle of *Hematodinium* species remains elusive, as positive findings by PCR have not been histologically substantiated (PagenkoppLohan et al. 2012).

In turn, *Syndinium* spp. are long known parasites of copepods and ciliates (Chaton 1910, Soyer 1974, Coats 1999, Scovgaard et al. 2005). Parasites in this lineage proliferate inside the haemal sinuses of copepods, forming a vast plasmodium, which feeds on host tissues and grows to eventually make internal organs and structures collapse (Ianora et al. 1990). The high mortality rates associated to the disease cause a severe impact on the populations of a number of susceptible copepod genera (Kimmerer & McKinnon 1990, Scovgaard & Saiz 2006). There is just a single instance of a *Syndinium*-like species infecting an amphipod host (Manier et al. 1971). The authors described the pathogen from explants of *Gammarus locusta* collected from the Mediterranean lagoon "Étang de Thau" (France).

Although the ultrastructure of what Manier et al. (1971) described as uni-nucleated plasmodia (15µm) is remindful of our uninucleated trophonts (6-8 µm), the configuration of condensed nuclear chromatin in the syndinian parasite infecting *Gammarus* sp. in Camel estuary is more similar to that of *Hematodinium* spp. Besides, we did not observe the fibrillar bodies present in plasmodia, trophonts and spores of *S. gammari*, although it must be noted that spores or plasmodia with more than two nuclei were not found, neither the characteristic "syndinian mitosis" (Ris & Kubai 1974, Soyer-Gobillard 2006). Nevertheless, our phylogenetic analysis using 5.8s rDNA (as ITS1 and ITS2 regions did not align to any known syndinian sequences) indicates

that the parasite infecting the *Gammarus* sp. population from Camel estuary is more closely related to the copepod-infecting genus *Syndinium* than to the decapod-infecting *Hematodinium* sp. While no records of *S. gammari* exist since 1971 and its DNA has never been sequenced, it appears reasonable to postulate that the dinoflagellate parasite infecting *Gammarus* sp. in Camel is indeed *S. gammari*. However, given the size and ultrastructural differences between cells, and the geographical distance between hosts, it is also possible that the parasite represents a novel species within an amphipod-infecting *Syndinium*-like lineage. After all, equivalent levels of molecular change account for phenotypical and geographical differences among *Syndinium* spp. infecting copepod hosts (Gomez 2012, Scovgaard 2014).

Apart from protists, other eukaryotes, prokaryotes, and viruses were microscopically detected infecting the amphipod population inhabiting Newton's Cove. While their histopathological, structural, and molecular analysis goes beyond the scope of this chapter, they are still considered as possible vectors, carriers, or co-occurring infections. Among metazoans, nematodes are the most prevalent, mostly associated/attached to carapace and pleopods, although occasionally they have been noticed inside the haemal sinuses and connective tissues. Actually, several families of nematodes, some of them human pathogens have been documented in amphipod hosts (Bush et al. 2012) and reviewed by Bojko & Ovcharecko(2019), including Anisakidae (*Anisakis* sp., *Pseudoterranova* sp., *Hysterothylacium* sp.) or Cystidicolidea (*Ascaropsis* sp., *Cystidicola* sp.). For instance, *Pseudoterranova decipiens,* a pathogen of humans, seals, and fish, has among others, *Echinogammarus* sp. and *Gammarus* sp. as intermediate hosts (Palm 1999). Similarly, *Ascarophis artica* has been documented in several *Gammarus* species. (Zander et al. 2002).

Digenetic trematodes are also common parasites in all three amphipod genera investigated in Newton's Cove, typically appearing imbedded in the dorsal skeletal muscle eliciting a strong host reaction including encapsulation, melanization and haemocytic infiltration. From the fourteen species confirmed to affect amphipods (Bojko & Ovcharenko 2019), three are known to infect *Echinogammarus* sp. and *Gammarus* sp.: *Coitocaeum angusticolle*, *Nicolla gallica*, *Pleurogenoides medians* (Lefebvre & Poulin 2005), which also cause infection in fish and amphibians. Occasionally copepod parasites were detected infesting the egg pouch/marsupium of amphipods in Newton's Cove, an ectoparasitic association that affects the fecundity of the host (Gotto & O'Connor 1980). Several copepod species, most of them belonging to genus *Sphaeronella* have been documented to infect amphipods (Costello & Myers 1989), but none are reported in *Echinogammarus* sp., *Gammarus* sp., or *Orchestia* sp.

Aside from metazoans, one more micro-eukaryote was identified infecting amphipods. The presence of yeast-like cellular structures indicates that it possibly belongs to Fungi, but molecular confirmation is still needed. Microscopically, the parasite, which infects connective tissues and haemal sinuses, is remindful of uncharacterized yeast-like diseases documented in *Diporeia* in the Great-Lakes (Messick et al. 2004, Kiziewicz & Nalepa 2008, Winters et al. 2014). Hardly any endoparasitic fungal infections have been documented in amphipods, and only *Candida gelida* and *Cryptococcus gammari* have been identified (Segerstråle1937, García et al. 2000). The impact of these infections in individual hosts and amphipod populations is yet to be determined (Bojko & Ovcharenko 2019) but should be addressed by future works (together with ultrastructure, phylogeny, host range, and cycle). Morphologically comparable parasites have been noticed to infect crabs from Newton's Cove and nearby locations in the southern British Isles (Stentiford et al. 2003, Davies et al. 2020).

Infections by non-eukaryotic parasites (bacteria and viruses) were also detected in amphipods from Newton's Cove, and their temporal variability examined, given their potential to impact the immune system, or as co-occurring parasites (Hurst & Darby 2009; Zindel et al. 2011). Infections caused by bacteria appeared mainly associated to connective, haemolymph, and gill tissues, while microscopic signs of viral infection were exclusively identified in the nuclei of gill cells. None of these infections was ultrastructurally or molecularly characterized, but several bacterial and viral infections are known to occur in the gills, connective, and haemolymph of gammarid and talitrid amphipods (Mengoni et al. 2013, Bojko et al. 2018).

4.4 Temporal variability and co-occurrence of amphipod-infecting parasites in Newton's Cove

Backed by the continuous improvement of DNA sequencing techniques and associated bioinformatic tools, molecular-based investigations are profoundly transforming our understanding of protistan communities and diversity (Caron & Hu 2019). Both processing and analysis are speeding substantively, consenting larger-scale temporal and spatial analyses (Wu et al. 2018, Gran-Stadniczeñko et al. 2019). In addition to environmental DNA, metagenomic investigations exploring the pathogens (eukaryotes, prokaryotes, and viruses) associated to a host, the pathobiome (Bass et al. 2019), are emerging on a regular basis (Gomez-Chiarri et al. 2015, Martínez-Porchas & Vargas-Albores 2017, Behringer et al. 2020, Holt et al. 2020). However, the concept of pathobiome, implicates reduced or potentially reduced health status of the host, rendering microscopy and other visual techniques indispensable to demonstrate association between molecular detection of pathogenic lineages and actual disease (Bass & Del Campo 2020, Bateman et al. 2020). As species with greater commercial interest get prioritized,

metagenomic approaches to the pathobiome of amphipods are exceptional and limited to specific lineages, mainly bacterial (Dattagupta et al. 2009, Abdelrhman et al. 2017). Equally, only few amphipod species have been histopathologically analysed (Chatterjee & Fernandez-Leborans 2013, Bojko et al. 2017, Bojko et al. 2019), seldom on a temporal scale (Messick et al. 2004). This seasonal analysis of amphipods *Echinogammarus* sp., *Gammarus* sp., and *Orchestia* sp., constitutes a baseline histopathological screening for these three genera. It also outlines the importance of seasonality as a variable to examine the occurrence of certain parasitic lineages in amphipods, and possibly other invertebrates. Additionally, it shows how parasites alternate between different amphipod species, often causing quick and virulent infections that can easily remain unregistered if not monitored, at least, on a monthly scale.

The application of a PCA summarizes reasonably well how temporal variability for certain parasitic infections is profoundly influenced by the host. For instance, ciliates have been observed to be the most prevalent protist lineage associated to amphipods, a finding that harmonises with environmental DNA studies, which count Ciliophora as one of the most abundant protist lineages in coastal ecosystems (Zhang et al. 2018, Boscaro et al. 2019). The incidence of ciliate epibionts/ectoparasites is very high in all three amphipods examined, ranging between 70% and 100%, doubling values observed in freshwater amphipod genera *Diporeia* or *Dikerogammarus* (Messick et al. 2004, Bojko et al. 2013). Epibiotic ciliates in amphipods have been shown to be more prevalent in marine than in freshwater habitats (Fenchel 1965), apparently due to higher planktonic biomass (Pitsch et al. 2019). In fact, while the PCA does not indicate inter-host variability, it suggests a higher prevalence during early summer, when the bacterial and micro-plankton concentration is higher in the western English Channel (Rodriguez et al. 2000). While plankton-mediated increase in the number of ciliates means that they are predominantly commensals rather than parasites, a more detailed molecular based identification could assist in identifying temporal changes in exclusively parasitic lineages (Prokopowicz et al. 2010, Chantangsi et al. 2013).

The incidence of gregarine endosymbionts/endoparasites in amphipod genera *Gammarus*, and *Orchestia*re mains rather steady throughout the year as well, with values ranging between 20% and 40%. In contrast, infection rates appear to fluctuate considerably in *Echinogammarus* sp. hosts, as outlined by the PCA. Metagenomic analyses have shown that gregarines infecting *Gammarus* sp. and *Echinogammarus* sp. belong to genus *Heliospora*, a gut-based parasite already known from *Gammarus* species. (Wróblewski et al. 2020). A two-year longitudinal study in *Gammarus fasciatus* (Grunberg & Sukhdeo 2017), observed that apart from a similar incidence (40-50%) and temporal variability, *Heliospora*-infected *G. fasciatus* were rarely affected by a second gregarine species at the same time. Additionally, host-size was

identified to be the main driver for differences in prevalence, with fully-grown adults being more frequently parasitized, regardless of population size. Furthermore, host size was one of the main factors driving prevalence in other gregarine-amphipod associations (Prokopowicz et al. 2010).

Based on this, higher *Heliospora* sp. prevalences in *Echinogammarus* sp. and *Gammarus* sp. would be expected when the ratio between adults and younger individuals is at its highest, say, just before females release their hatch (young full developed amphipods until then surviving in the egg pouch). While our current data are not detailed enough to link the gregarine prevalence with host size in amphipods from Newton's Cove, the four infection peaks observed on alternate months in *Echinogammarus* sp. correspond with the duration and annual number of generations characteristic of this genus (Marques & Nogueira 1991, Maranhão & Marques2003), suggesting a connection with the host's reproductive cycle. In fact, the infection prevalence observed for epibiotic nematodes in amphipods, which occurrence has been shown to be negatively associated to a moulting carapace (Hudson & Lester 1994), further supports this link. The incidence of nematodes appears to fluctuate in a similar time scale to that of gregarines, and moulting episodes are known to be directly related to the reproductive cycle in amphipods. Since only a new and flexible cuticle allows the migration of unfertilized eggs to the egg-pouch (Sutcliffe 1992).This rationale is also consistent with nematodes associated to *Gammarus* sp., whose prevalence in the population crashes twice, overlapping with the two breeding peaks noticed in Newton's Cove *Gammarus* sp. population. A bi-modal reproductive cycle common among marine *Gammarus* spp. (Sutcliffe 1993).

In contrast to Ciliophora, Gregarinasina, and Metazoans (nematodes, copepods, digeneans), which in spite of some seasonal variability, appear associated to amphipods all year long, the incidence of filasterean, haplosporidian, microsporidian, and paramyxid parasites differs substantially based on host species and season. In fact, the PCA shows a clear difference between the vigorous and short-lived microcell-caused diseases in *Echinogammarus* sp. versus the steadier development observed in genera *Gammarus* and *Orchestia*. Ascetosporean lineages Haplosporida and Paramyxida effectively exemplify this dichotomy. Both, *Haplosporidium echinogammari* and *Paramarteilia orchestiae*, infect between 5% and 15% of *Gammarus* sp. individuals virtually all year long. In contrast, *H. echinogammari* and *P. orchestiae* only infect *Echinogammarus* sp. in June and August/September respectively, when a significant part of the amphipod population is infected, especially in the case of the haplosporidian. These incidences (74% for *H. echinogammari*; 21% *P. orchestiae*) are unusually high for *Haplosporidium* sp. and *Paramartielia* sp. infecting amphipods and other crustaceans (Feist et al. 2009, Winters & Faisal 2014, Davies et al. 2020), which normally range between 1% and 3%, except for *H. littoralis* (7.6 %) infecting crabs (Stentiford et al. 2013).

While high prevalence could easily be explained by a population density several orders of magnitude higher in *Echinogammarus* sp., than in the two other amphipod species, not only in Newton's Cove but in coastal ecosystems all over Europe (Persson 1999, Martins et al. 2002), identifying which factors determine the marked seasonality of ascetosporean infections in *Echinogammarus* is far more complex. Somehow, *Echinogammarus* sp. amphipods inhabiting the upper zone of the intertidal zone, enter into contact either with spores or infected low-intertidal *Gammarus* sp. individuals during late spring/early summer and get infected by *H. echinogammari*. Then, the parasite reproduces unreservedly in the host's haemolymph and connective tissues until it is transmitted, most likely horizontally (heavy systemic infection, speedy transmission, larvae not infected), to other amphipod hosts in the population. Following this quick disease outbreak, the parasite virtually disappears from the *Echinogammarus* sp. population by late July/August possibly to infect a further host, as spore formation has not been noticed. The development of paramyxid-mediated infections exclusively during late summer in *Echinogammarus* sp. and *Orchestia* sp. is even more intriguing, as the parasite is supposed to be predominantly vertically transmitted (Ward et al. 2016, Guler et al. 2018). Besides, microcells are not detected in *Gammarus* sp., the main reservoir, from April to late summer, rendering more difficult infection by direct contact between amphipod species, and supporting the existence of additional intermediate hosts.

Factors influencing temporal variability of ascetosporean infections have only been investigated for commercially significant bivalves (Haskin & Andrews 1988, Robledo & Figueras 1995, Burreson & Ford 2004, Albuixech-Martí et al. 2020). For instance, investigations on *Haplosporidum* spp., *Bonamia* spp., *Minchinia* spp., or *Marteilia* spp. infecting oysters, cockles, and mussels, have shown that temporal variation of abiotic factors such as temperature or salinity alone do not explain disease outbreaks (Burreson & Ford 2004, Laing et al. 2014). Furthermore, studies usually discuss the need of intermediate hosts or vectors to explain the completion of the parasite's life cycle and its seasonal variation (Arzul et al. 2014). Although, conclusive data are still needed, we have histological evidence of ciliates carrying numerous haplosporidian cells (Fig. 17) and PCR-based molecular proves of harpacticoid copepods either infected or transporting *Haplosporidium* sp. cells (Fig. 18). Moreover, both ciliates and harpacticoid copepods have been shown to be especially prevalent during early summer, coinciding with the haplosporidian-infection outbreak in *Echinogammarus* sp. While further work is needed to confirm ciliates and/or copepods as possible vectors or intermediate hosts, high infection rates suggest that amphipods are not opportunistic hosts, but important reservoirs of ascetosporean parasites, with *Gammarus* sp. working as a long-term pool and *Echinogammarus* as a seasonal amplifier.

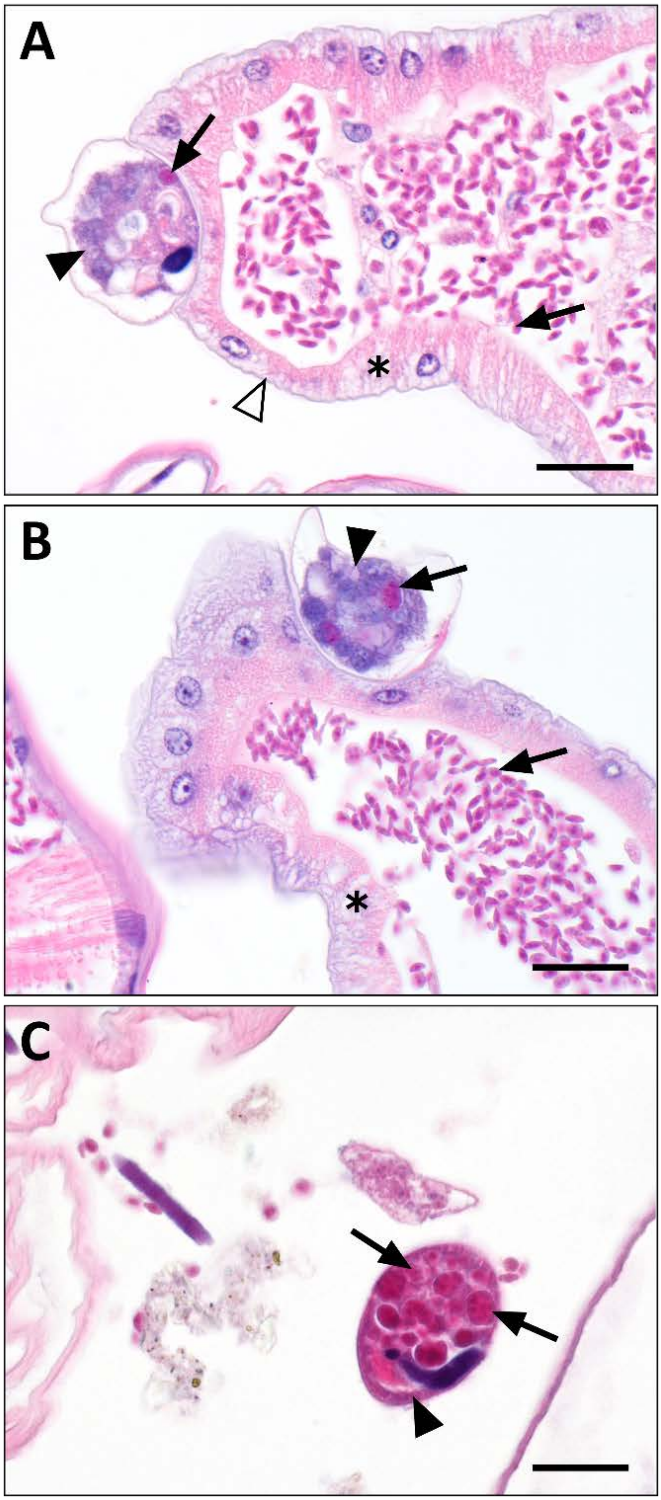


Figure 17: Light microscopic images of *Haplosporidium* sp. infecting amphipods and possibly amphipod-associated ciliates. **(A, B)** Cells of *Haplosporidium orchestiae* (arrow), congest the haemal sinuses in the gills (asterisk) of amphipod *Orchestia* sp. Notice stages of the haplosporidian parasite inside epibiotic ciliates (arrowhead) that were attached penetrating the gill cuticle (empty arrowhead) of the amphipod. **(C)** Detached epibiotic ciliate (arrowhead) presents dozens of *Haplosporidium* sp. cells (arrows) inside. Scale bars for A,B, C = 20 µm.

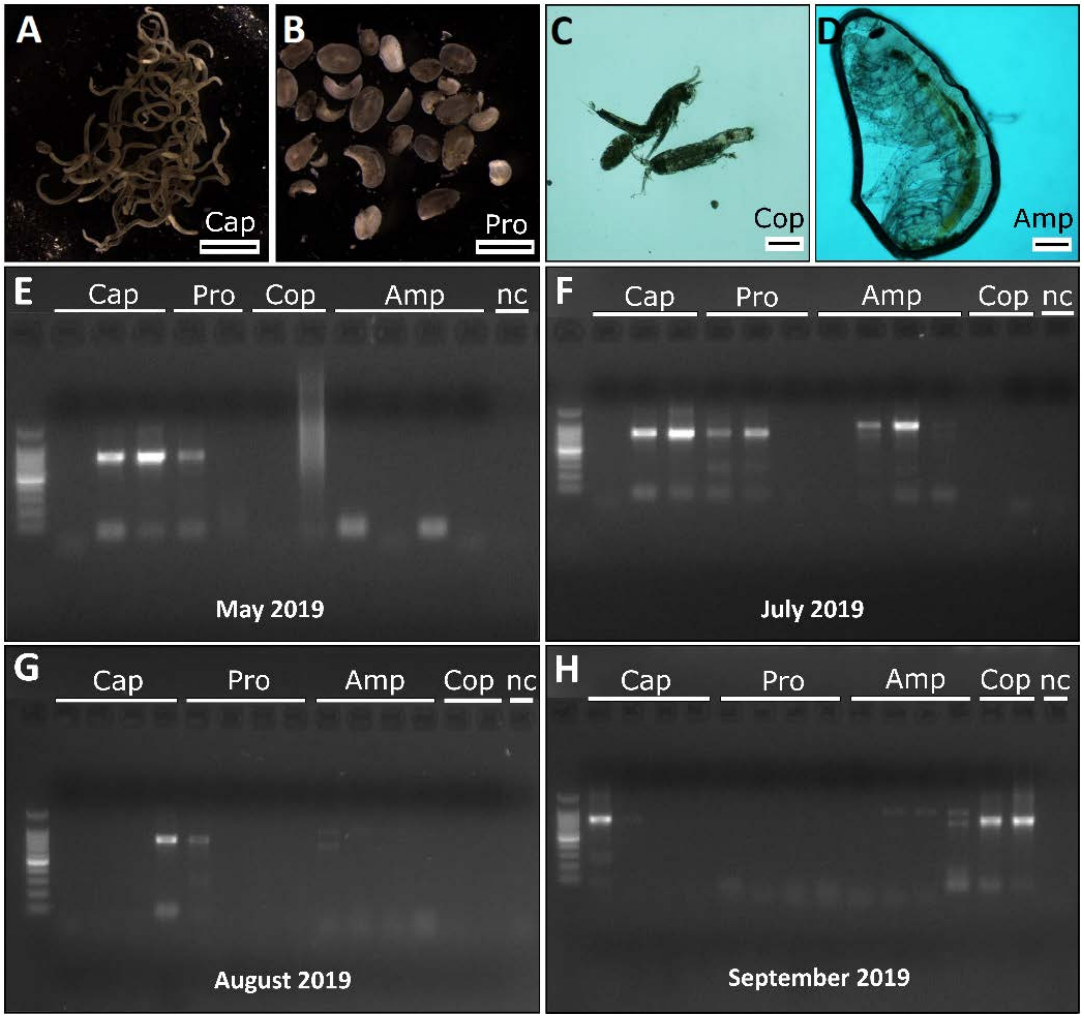


Figure 18: Gel electrophoresis of PCR amplified 18s rRNA fragments using *Haplosporidium* sp. specific primers on batches of individuals pertaining to the four organisms shown in the upper part of the figure. The organisms were collected from the upper part of the intertidal in Newton's Cove as specified in (Table 1) and are represented as: (A) *Capitella* sp. (B) *Procerodes* sp. (C) Harpacticoid copepods. (D) *Echinogammarus* sp. The results for the PCR runs shown by gel electrophoresis for the different months of samplings (E, F, G, H). Cap = *Capitella* sp., Pro = *Procerodes* sp. Amp = *Echinogammarus* sp. Cop = Harpacticoid copepods. nc = negative control. The steps of the ladder shown in the left of the gels are 100 bp.

Regarding the temporal variability of opisthokonts, *Txikispora philomaios* represents the first confirmed parasite in Class Filasterea, rendering difficult comparison with related parasitic species (discussed in Chapter 3). The small amoeba has been observed infecting genera *Echinogammarus* and *Orchestia*, but not *Gammarus*. Infection in Newton's Cove occurs almost exclusively during late April and May, being significantly higher (40-65%) in *Echinogammarus* sp. than in *Orchestia* sp. (~10 %) ,possibly due to higher population density in the gammarid (Martins et al. 2002). However, a higher incidence could be explained by different feeding strategies or host susceptibility, as both amphipods inhabit the same habitat in the upper part of the

intertidal. Feeding mechanisms in *Echinogammarus* sp. include scavenging and predation (Dick et al. 2005, Alexander et al. 2012), favouring transmission by ingestion of infected prey/corpses. Besides, species in this genus are known to have high levels of cannibalism (Maranhão & Marques 2003), bolstering a possible horizontal transmission. In contrast, *Orchestia* spp. are detritivorous/herbivorous, feeding predominately on algae (Moore & Francis 1985, Hines & Denno 2007), which combined to lower population densities might explain diminished *T. philomaios* prevalence.

The network analysis of co-occurrence between parasites conducted in *Echinogammarus* sp. hosts has shown that individuals parasitized by *T. philomaios* often present *Haplosporidium* sp. infections, as well. The co-infection occurrence is greater than expected by chance (Pearson's Chy-squared test for independence, $p < 0.001$), although the reasons for it remain unknown. In other parasitic lineages such as Microsporidia, Myxozoa, or Coccidiasina, co-infection might be result of hyper-parasitism (Gómez-Couso et al. 2007, Stentiford et al. 2017, Sokolova & Overstreet 2020), a compromised immune system (Supali et al. 2010), or sharing a common vector/intermediate host (Poulin et al. 2003). So far, there is no microscopic evidence of hyper-parasitism, and potential intermediate hosts/vectors are yet to be confirmed for both parasites. Future work including transcriptomics might reveal a down-regulation of genes involved in the normal functioning of the immune system in infected individuals.

Analysing the temporal variability of microsporidian infections affecting the amphipod population in Newton's Cove and the driving factors, is further complicated because at least two different *Dictyocoela* sp. have been phylogenetically and morphologically demonstrated in the location. Infection in *Echinogammarus* sp. is caused by *D. duebenum*, although *E. marinus*, *E. berilloni* and *E. trichiatus* populations inhabiting coastal ecosystems in the English Channel have been shown to host *D. berillonum* as well (Terry et al. 2004, Bacela-Spychlaska et al. 2018). Actually, infections by both *Dictyocoela* sp., can overlap in time and space in the same amphipod population (Wilkinson et al. 2011). One way or another, the incidence of *Dictyocoela*-infections appears to be especially prevalent in *Echinogammarus* sp. during late summer (August/September), coinciding with infection by paramyxid microcells. Additionally, the association network analysis indicates that both parasites tend to happen in the same host organism, a co-infection occurring more frequently than expected by chance (Pearson's Chy-squared test for independence, $p < 0.001$).

Unlike co-infection by *Haplosporidium* sp. and *Txikispora* sp., this co-occurrence between microsporidian and paramyxid parasites is widely documented (Villalba et al. 1997,

Short et al. 2012). Furthermore, *Dictyocoela* sp. and *Paramarteilia* sp. parasites have been linked to feminization of infected amphipods, possibly as a way of increasing vertical transmission (Ginsburger-vogel 1991, Ironside et al. 2003, Ironside & Alexander 2015). However, more recently, the feminising effect has been linked exclusively to the paramyxid (Pickup & Ironside 2018, Guler et al. 2018). An equivalent co-infection, between the microsporidian *Hyperspora aquatica* and the paramyxid *Marteilia cochillia*, has been shown to be result of hyper-parasitism with microcells of *H. aquatica* hitchhiking into its final host, the cockle *Cerastoderma edule*, inside *M. cochillia* spores (Villalba et al 2014, Stentiford et al. 2017). However, based on the microscopical analysis, *D. duebenum* does not appear to hyper-parasitize *P. orchestiae* to hitchhike into *Echinogammarus* sp., a finding shared by Guler et al. (2018) based on a study conducted on *E. marinus* collected throughout the British Isles.

Except for the ovaries, infected by both parasites, the microsporidian and the paramyxid have a different tissue tropism. Hyper-parasitism discarded, it has been proposed that *D. duebenum*, and *P. orchestiae* might modulate their transcriptomic output to accommodate the presence of their co-infecting partner (Guler et al. 2018). Although, the peak of microsporidian and paramyxid co-infection does not seem to be associated to a significant increase in the ratio of intersex individuals, the decrease in the number of larvae-bearing females is unquestionable. A larvae-hatching collapse produced by female castration would not make much sense for vertically transmitted parasites, a predominant strategy among microsporidians and paramyxids parasites (Dunn et al. 2001, Ward et al. 2016). However, the co-infection is not associated to sex ratio distortion either, turning down a potential explanation by incomplete feminization of males (Cormier et al. 2021). The high incidence of heavy systemic *D. duebenum* infections in *E. marinus* during this period, and the reduced number of larvae, could suggest that the microsporidian is changing transmission strategy from vertical to horizontal, maybe to infect other hosts rather than *Echinogammarus sp.* An analogous course of action has already been documented for other species in the phylum (Haag et al. 2020). A deadlier stage of horizontal transmission in *D. duebenum* (Guler et al. 2018) could explain the reduction in the number of amphipod larvae and consequently the end of vertically transmitted *P. orchestiae* infections observed, although more data are needed to substantiate this hypothesis.

In turn, microsporidian infections in *Orchestia* sp., which according to our phylogenetic analysis are most likely caused exclusively by *D. cavimanum*, occur throughout the year, with incidence values around 20%. This level of incidence is identical to the one observed by Terry et al. (2004) for *D. cavimanum* infecting amphipod *Orchestia cavimana* collected in Scotland, and considerably higher than the incidence of sister lineage *D. gamarellum* parasitizing *O.*

Gammarellus (4-5%). While the feminising effect observed for this microsporidian species (Terry et al.2004) has not been documented further, it appears to be associated to high levels (up to 20%) of intersex individuals in *Orchestia* sp. Additionally, the association network analysis indicates that co-infection by *D. cavimanum* and *P. orchestiae* is likely, as in *Echinogammarus* sp. (Pearson's Chy-squared test for independence, $p < 0.001$). An equivalent connection is less clear in *Gammarus* sp. hosts (Pearson's Chy-squared test for independence, $p = 0.0031$), possibly because the genus has been shown to be infected by several *Dictyocoela* spp. including *D. duebenum*, *D. muelleri*, *D. roeselum* and *D. berillonum*. Consequently, the difficulty to explain the great incidence variability (5-45%) for microsporidian infections in *Gammarus* sp. This amphipod likely behaves as reservoir for several *Dictyocoela* spp. in Newton's Cove.

The incidence of oomycete and fungal infections in amphipods from Newton's Cove is low (< 5%), suggesting that these three amphipod genera are opportunistic hosts rather than reservoirs. However, it is possible that oomycete and fungal parasites are more prevalent in other amphipod species present in Newton's Cove, or other coastal areas in southwest England. So far, the taxonomy of these parasites remains unknown, but the occurrence of oomycete and fungal infections in crabs and lobsters in this area of the English Channel (Stentiford et al. 2003, James et al. 2017, Holt et al. 2018, Davies et al. 2020) advice for a detailed phylogenetic study and more screenings. In case that the amphipods represent intermediate hosts or vectors for these lineages, as hypothesized by Sarowar et al. (2013), Svoboda et al. (2014), or Bojko & Ovcharencko (2019).

4.5 Relating incidence and temporal variability of amphipod-associated parasitesfrom Newton's Cove to other locations in the western English Channel.

Infections caused by micro-eukaryotes in the amphipod community from Newton's Cove have been shown to be governed by the time of the year, and to progress differently in each host. Seasonal differences in the incidence of protist parasites are particularly evident in host *Echinogammarus* sp., which apart from being one of the most abundant organisms in Newton's Cove and coastal locations all over Europe (Maranhão et al. 2001), forms densely packed assemblages in the upper intertidal zone. While essentially affected by the same parasitic lineages, infections in *Gammarus* sp. and *Orchestia* sp. have a steadier progress throughout the year, possibly due to a distinct diet, more sparsely distributed populations, or differences in susceptibility. Aside from analysing possible reasons for this dichotomy, assessing whether these

infections, their prevalence, and seasonal occurrence are extrapolatable to other estuarine and coastal locations in the western English Channel is essential.

Alveolate clades Ciliophora and Gregarinasina remain the most abundant protist lineages associated to amphipods, although their prevalence varies between locations and seasons, especially for gregarines. In the case of Ciliophora, the main difference between amphipods from Newton's Cove and populations inhabiting Tamar, Dart, and Camel estuaries, is that in the later, ciliates are more abundant in spring (April) than in summer (June). In the above sections (4.3 and 4.4), the amphipod-ciliate association has been discussed to be predominantly epibiotic (although the cuticle is often penetrated), and higher prevalences have been linked to increased levels of phytoplankton and bacteria in the sediment (Pitsch et al. 2019). While higher concentration levels of primary producers, including blooms, are largely site-specific (Iriarte & Purdie 2004, Carstensen et al. 2015), estuaries in the south of England usually have earlier and more exuberant heights than exposed coastal habitats (Kocum et al. 2002, Cloern et al. 2014). Therefore, a premature peak of ciliate infection in estuaries is consistent with the previously exposed rationale for temporal differences in the prevalence. Besides, almost all amphipod-associated ciliate genera identified in amphipods from Newton's Cove belong to Classes Phylopharingea and Oligohymenophorea, which are considerably more abundant in estuarine waters than in euhaline coastal habitats (Urrutxurtu et al. 2003, Sun et al. 2017).

In turn, the prevalence of gregarines infecting estuarine amphipod populations is substantially higher than the one observed in amphipods inhabiting the coast. Environmental DNA studies have shown that diversity and abundance of apicomplexan OTUs is greater in estuarine and coastal ecosystems than in freshwater habitats (Bazin et al. 2014, Rueckert et al. 2011), but differences between euhaline and polyhaline ecosystems are largely genus-dependant (del Campo et al. 2018). So far, only a handful of works have analysed host-associated gregarine prevalence on a spatial level, for the most part in commercially important invertebrate clades including crabs (Messick et al. 1998), cockles (Carballal et al. 2001), or oysters (Winstead et al. 2004). However, spatial variability in the prevalence of amphipod-infecting gregarines remains undocumented. One of the better studied gregarine genera is *Nematopsis*, which has been shown to be negatively correlated to higher salinities, in *Mytilus* sp. mussels (Kovačić & Pustijanac 2017), and *Litopennaeus* sp. shrimps (Jimenez et al. 2002). A comparable behaviour for amphipod-infecting gregarines would back up the higher prevalence observed in estuarine ecosystems. In the above sections, the prevalence of amphipod-infecting gregarines in Newton's Cove has been discussed to be positively correlated to a larger host size (Prokopowicz et al. 2010,

Grunberg & Sukhdeo 2017). Consequently, a greater incidence might be expected in ecosystems where same-species amphipods grow bigger. While attempting to not oversimplify a certainly multivariate issue, the slower growth and bigger sizes observed for all three amphipod species in estuaries (Maranhão & Marques 2003) is so far consistent with the size-related hypothesis as well.

Protist lineages Microsporidia and Paramyxida, cause infections in all amphipod genera and locations investigated. Their occurrence and prevalence explain, together with clade Gregarinasina, most of the variability observed between estuaries and Newton's cove. Both parasites, which are especially prevalent during late summer, appear to be more abundant in estuaries than in coastal habitats. Furthermore, fundamentally identical microsporidian and paramyxid infection prevalences in *Echinogammarus* sp. regardless of season and location substantiate the co-occurrence of both parasites discussed in the above section (4.4). This association has been amply investigated but remains mostly uncharacterized in a temporal and geographical basis (Terry et al. 2004, Wilkinson et al. 2011, Ironside & Pickup 2015, Guler et al. 2018). Based on our findings, the co-infection, which in *Echinogammarus* sp. from Newton's Cove is restricted to late summer, is clearly bimodal in estuarine populations. Differences in the population dynamics of the amphipod between estuaries and coastal habitats, or in the occurrence of intermediate hosts, might explain the existence of this second peak of co-infection during winter in estuaries. Actually, both factors have been suggested to influence temporal and spatial variability in other paramyxids, such as *Marteilia* spp. (Audemard et al. 2002, Carrasco et al. 2007) and microsporidian species, including *Dictyocoela* (Guler et al. 2018). However, assuming the existence of alternation between transmission strategies as proposed by (Grabner et al. 2015), a disparity in the transmission strategy between estuaries and coastal habitats should not be ruled out. Whichever the reasons for this variability in the disease prevalence, our findings suggest that unlike in Newton's Cove, estuarine *Echinogammarus* sp. populations represent a reservoir of *Paramarteilia orchestiae* and *Dictyocoela* sp. all year long.

The statistical analysis has shown certain level of co-infection by *Dictyocoela* sp., and *Paramarteilia* sp. in the other two amphipod genera investigated (*Gammarus* and *Orchestia*) as well, but infections do not co-occur as markedly as in *Echinogammarus* sp. In fact, both parasites are known to cause independent infections in these and other amphipod and crustacean species (Bacela-Spychalska et al. 2018). A handful of studies have attempted to explain spatial and temporal variability for *Dictyocoela* spp. (Ryan & Kohler 2010, Quiles et al. 2020). For instance, incidence has been shown to be correlated to temperature for *D. duebenum* infecting amphipod *Gammarus duebeni* in the Isle of Mann, with lower temperatures inhibiting the parasite's

replication (Dunn et al. 2006). Although this finding would be consistent with maximum infection prevalence observed for *Echinogammarus* sp. and *Gammarus* sp. in Newton's Cove during late summer (when water temperature is at its highest);it would fail to explain the second infection peak observed during winter in estuaries, at least in *Echinogammarus* sp. Temperature has been shown to be lower in Tamar, Dart, and Camel estuaries than in coastal locations nearby (Uncles & Stephen 2001, Thain et al. 2004).

In turn, existing data regarding the spatial and temporal variability of *Paramarteilia*-caused infections in crustaceans (amphipods and crabs) are limited (Feist et al. 2009, Short et al. 2012, Ward et al. 2016). The thorough monthly screening (n = 686) of *P. canceri* infecting *Cancer pagurus* (Feist et al. 2009) in coastal locations around Dorset (South England), showed prevalence to be higher (3%) during winter, but the low incidence prevented spatial comparisons. Following, Ward et al. (2016) observed paramyxid infections in amphipods (*Orchestia gammarellus*), to be higher in the Gann estuary (5.58%, n=197) than in a coastal location near Newton's Cove (0.56%, n=178). While limited to two locations sampled in different months (as it was beyond the scope of their phylogenetic investigation and review of the order), their results are consistent with findings by the present study, showing a preference of *P. orchestiae* for estuarine amphipod populations or estuarine conditions. This putative inclination for estuarine habitats is possibly shared by related genus *Marteilia* as well, observed to be more prevalent in mussels collected in estuaries (Tamar), than in coastal locations nearby (Bignell et al. 2011). On the one hand, it is evident that a better comprehension of *Paramarteilia* sp. cycle will be necessary to grasp the drivers influencing the appearance and development of the disease in amphipods, crabs, and maybe other invertebrate hosts. On the other hand, our results advise against analysing these two infections (Microsporidia and Paramyxida) separately in populations where co-infection has been documented or remains undetermined, as the mechanisms driving co-occurrence are not fully comprehended yet (Guler et al. 2018).

In line with ciliates and gregarines, haplosporidian infections appear to surge earlier in amphipods inhabiting estuaries than in Newton's Cove. For instance, *Haplosporidium echinogammari*, which is almost exclusively observed during June in the *Echinogammarus* sp. population from Newton's Cove, occurs in estuarine populations throughout the year, with infections peaking earlier during spring. In turn, *Haplosporidium orchestiae,* which is not present in coastal waters (Newton's Cove), infects *Orchestia* sp. populations in all three estuaries analysed, being more prevalent during the first half of the year. Until the description of *H. echinogammari* and *H. orchestiae* (Urrutia et al. 2019), the only amphipod-infecting haplosporidian species described was *Haplosporidium diporeiae*, which causes disease in the

freshwater genus *Diporeia* from the Great Lakes (USA). However, no spatial trends have been observed in the distribution of this parasite in its type-location (Winters & Faisal 2014). Similarly, the other three *Haplosporidium* spp. causing infections in crustaceans, *H. littoralis*, *H. carcini*, and *H. cranc*, are only known from their type locations in the British Isles (Stentiford et al. 2013, Davies et al. 2020).

In contrast, the prevalence, infection intensity, and lethality of *H. nelsoni*, a long-known pathogen of oysters thoughtfully researched through time and space, are known to be primarily regulated, by salinity and to lesser extent by temperature (Ford & Haskin 1988, Carnegie & Burreson2011). The parasite, in consonance with *H. echinogammari* has been noticed to be especially prevalent during early summer, when water temperature is increasing (Ford 1985). Additionally, *H. nelsoni* has been shown to be especially prevalent in estuarine waters as well, possibly driven by optimal levels of salinity and a higher concentration of infective stages (Barber et al. 1997). Anyhow, the above discussed hypothesis of ciliates and/or copepods behaving as vectors or intermediate hosts (yet to be confirmed ultrastructurally) gets substantiated by this premature peak of haplosporidian infections in estuaries, as it co-occurs with observed ciliate and zooplankton heights (Kocum et al. 2002, Cloern et al. 2014).

The presence of the novel amphipod-infecting parasite *T. philomaios* is not restricted to Newton's Cove. In fact, infections caused by the filasterean, which in Weymouth occur almost exclusively during May, have been microscopically detected throughout the year in Tamar and Dart estuaries, although its predominant prevalence during Spring is evident in the PCA. However, the quarterly analysis conducted in estuaries does not allow addressing some important questions, such as the existence in estuaries of equivalent annual outbreaks to those observed in Newton's Cove, or the reasons for infection prevalence to be higher in estuarine populations of *Orchestia* sp. than those of *Echinogammarus* sp. when Newton's Cove Orchestia sp. was uninfected. These and other questions regarding transmission method, prevalence, distribution, or intermediate hosts are further discussed in Chapter 2.

The findings above outline the rapid shifts in the occurrence of some protist parasites, whose temporal distribution is being increasingly documented by molecular methods but seldom associated to infection (Berdjeb et al. 2018, Sassenhagen et al. 2020). The rapid generation time of some protists, in some cases spanning less than a day (Ohtsuka et al. 2016), promotes rapid swings in their prevalence. These ephemeral populations, which might last between one and three weeks (Vigil et al. 2009) constitute an evident bias for all but recurrent temporal analyses (Simon et al. 2015).

In general, the community of amphipod-infecting protist parasites observed in Newton's Cove appears to be representative of the main micro-eukaryotic infections affecting amphipods inhabiting marine and estuarine ecosystems in the southwest coast of England. The *Syndinium*-like parasite infecting *Gammarus* sp., which has only been detected during spring in the Tamar estuary, might represent the only exception. While this dinoflagellate could possibly be *Syndinium gammari* (Manier et al. 1971), a parasite undetected for 50 years, some ultrastructural, morphometric, and biogeographic differences, do not allow discarding a novel *Syndinium* species. Unlike then, DNA of the parasite is available now, and specific primers are being currently designed to investigate its occurrence in other locations and hosts. Closely related to *Hematodinium* sp., this *Syndinium* sp. (or *Syndinium* sp.-like) amphipod-infecting protist is most likely an obligate parasite as well (Stentiford & Shields 2005, Guillou et al. 2008). Although rare, its prevalence is considerably higher than that of opportunistic parasites, which tend to infect diseased or immunocompromised hosts (Mitchell & Rodger 2011). Further work will be necessary to understand if amphipods are casual, intermediate, or final hosts; the whereabouts of the parasite when is not infecting *Gammarus* sp.; the spatial and temporal prevalence of the parasite beyond the Tamar estuary during spring; and the susceptibility of other amphipod and crustacean species.

Considering all locations and seasons, populations of *Echinogammarus* sp. are slightly more parasitized than those of *Orchestia* sp., and considerably more parasitized than those of *Gammarus* sp., a difference that is statistically significant (p-value < 0.05, One-way Anova; $p < 0.05$, Tukey HSD test) when micro and macro-eukaryotic parasites are considered. The reduced number of general screenings for parasites in amphipods (Winters et al. 2014, Bojko et al. 2017), anticipate the lack of comparable studies analysing the parasitic load by host in this clade, a paucity that is extensive to other crustacean hosts as well (Stentiford & Feist 2005, Wolinska et al. 2011). Possibly, because the myriad of existing drivers (size, host ratio, diet, immune system, population density, etc.) renders interpretation notably complex (Vestbo et al. 2019). However, differences in the parasitisation level between individual hosts, populations, or lineages have been shown to be determinant in the distribution of certain genotypes, populations, and species at all possible spatial scales (Poulin & Morand 2000), outlining the value for its consideration and investigation.

For instance, the influence of parasitism in the distribution of a host at small spatial scale (few centimetres) is provided by talitrid amphipods, which have been shown to sustain higher incidences of metazoan parasites as they burrow deeper in the substrate (Poulin & Latham 2002). Apparently, burrowing depth is the result of a trade-off between increased exposition to

predators/desiccation and sustaining more parasites. The extrapolation of this individual-based hypothesis to *Orchestia* sp., *Echinogammarus* sp., and *Gammarus* sp. populations could explain the higher parasitic load observed in the first two. Both upper intertidal genera, are documented burrowers (Rossano et al. 2008), a behaviour frequently observed in Newton's Cove. In contrast, *Gammarus* spp. inhabiting the lower intertidal zone do not display such burrowing behaviour, as they have reduced pressure to tackle desiccation and can avoid predators more easily among algal fronds (Aikins & Kikuchi 2001). However, it is evident that further work considering more species and locations is needed to substantiate this hypothesis or alternative explanations. The differential distribution of parasitic spores and/or intermediate hosts between upper and lower intertidal zones could easily explain our observations as well (Hall et al. 2005, de Montaudouin et al. 2012); not to mention the above discussed inter-specific variability in diet, sex-ratio, immunity, age, or reproductive cycle among many other variables. In turn, the impact of parasitic load in the spatial distribution of a host population at large-scale is well illustrated by invasive species. There is mounting evidence demonstrating the weight of the pathobiome in the success, or failure, of species expanding their range or invading different continents (Gendron et al. 2012, Young et al. 2017, Lagrue 2017), amphipods included (MacNeil et al. 2003, Prenter et al. 2004, Kestrup et al. 2011).

There is no significant difference in the total pathogen load between seasons, when estuarine and coastal populations of the three amphipod genera investigated are grouped together (p-value > 0.05, One-way Anova). This does not mean that there are no differences in the occurrence and prevalence of certain amphipod-infecting protists clades between seasons, as above discussed (section 4.4). Our findings indicate that general or specific screenings for protist parasites could be especially biased during spring and summer, when communities change more and quicker in amphipods, and in the environment (Berdjeb et al. 2018) outlining the need for additional sampling effort during this time of the year. Our data also show an apparent "stability" in the seasonal parasitic load sustained by amphipods in this area of the UK. While clearly insufficient to draw equivalent hypotheses or explanations, these results call to mind those formulated by modelling in archetypal studies (Anderson & May 1978, Anderson & Gordon 1982), in which stabilized host populations might be in equilibrium with their parasitic burden given some conditions, including parasites exerting mortality.

Predominantly driven by higher incidences of microsporidian, paramyxid, and gregarine microcell parasites and nematodes, amphipods from estuarine waters are significantly more parasitized than those from coastal waters (p-value < 0.05, Kruskal-Wallis test; p < 0.05, pairwise Wilcox test). In fact, differences (Bray-Curtis dissimilarity) in the occurrence and prevalence of

protist parasites allow to distinguish (with certain overlap) between estuarine and coastal (Newton's Cove) host populations. Comparable results have been shown to consent a fine-scale spatial assessment of distribution and migration routes in fish populations (Levy et al. 2019, Lennox et al. 2020). Furthermore, equivalent histopathological analyses of other significant macrobenthic species, could procure a vision of the general health status and host-stress in different locations (Stentiford et al. 2003, Stentiford & Feist 2005). Insights that would certainly have an impact environmental assessment studies (Lafferty 1997, Marcogliese & Pietrock 2011) and influence decision making in fields ranging from aquaculture, and feeding industry to harbour/inner water management, including dredging, river transfers, or ballast waters.

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CHAPTER 2

Txikispora philomaios n. sp., n. g., a Micro-Eukaryotic Pathogen of Amphipods, Reveals Parasitism and Hidden Diversity in Class Filasterea

Abstract

This study provides a morphological, ultrastructural, and phylogenetic characterization of a novel micro-eukaryotic parasite (2.3-2.6 µm) infecting amphipod genera *Echinogammarus* and *Orchestia*. Longitudinal studies across two years revealed that infection prevalence peaked in late April and May, reaching 64% in *Echinogammarus* sp. and 15% in *Orchestia* sp., but was seldom detected during the rest of the year. The parasite infected predominantly haemolymph, connective tissue, tegument, and gonad, although hepatopancreas and nervous tissue were affected in heavier infections, eliciting melanization and granuloma formation. Cell division occurred inside walled parasitic cysts, often within host haemocytes, resulting in haemolymph congestion. Small subunit (18S) rRNA gene phylogenies including related environmental sequences placed the novel parasite as a highly divergent lineage within Class Filasterea, which together with Choanoflagellatea represent the closest protistan relatives of Metazoa. This phylogenetic position as the earliest branching filasterean was further substantiated by a phylogenomic analysis including a 96 protein-coding multi-gene tree. We describe the new parasite as *Txikispora philomaios* n. sp. n. g., the first confirmed parasitic filasterean lineage, which otherwise comprises four free-living flagellates and a rarely observed endosymbiont of snails. Lineage-specific PCR probing of other hosts and surrounding environments only detected *T. philomaios* in the platyhelminth *Procerodes* sp. We expand the known diversity of Filasterea by targeted searches of metagenomic datasets, resulting in 13 previously unknown lineages from environmental samples.

Keywords: *Echinogammarus*; *Orchestia*; Holozoa; Histopathology, Intracellular parasite; Haemolymph congestion; Environmental DNA.

1. Introduction

The Class Filasterea Cavalier-Smith 2008 currently comprises five species (Shalchian-Tabrizi et al. 2008; Hehenberger et al. 2017; Tikhonenkov et al. 2020). Initially classified as a nucleariid, *Capsaspora owczarzaki* was the first filasterean to be described (Stibbs et al. 1979; Owczarzak et al. 1980; Amaral-Zettler et al. 2001; Hertel et al. 2002; Ruiz-Trillo et al. 2004). This filopodial amoeba is a facultative endosymbiont (Harcet et al. 2016) isolated from explanted pericardial sacs of laboratory-grown *Biomphalaria* sp. snails (Stibbs et al. 1979; Morgan et al. 2002), which remains elusive in environmental samplings (Hertel et al. 2004; del Campo & Ruiz-Trillo 2013; Shanan et al. 2015; Ferrer-Bonet & Ruiz-Trillo 2017; Arroyo et al. 2018). In contrast, the other four species (*Ministeria vibran*s, *Ministeria marisola*, *Pigoraptor chileana*, and *Pigoraptor vietnamica*) are free-living flagellates, sampled from marine and freshwater ecosystems (Patterson et al. 1993; Tong et al. 1997; Hehenberger et al. 2017; Mylnikov et al. 2019). The discovery of *C. owczarzaki* drew considerable scientific attention, as resistant cysts present in the mantle of *Biomphalaria glabrata* were observed to attack and kill sporocysts of the trematode *Schistosoma mansoni* parasitizing the snail (Stibbs et al. 1979; Eveland & Haseeb 2011). *S. mansoni*, which has *B. glabrata* as intermediate host, causes schistosomiasis in humans, a disease affecting over 230 million people worldwide (Colley et al. 2014).

Filasterea are also of interest (Ruiz-Trillo et al. 2008; Suga et al. 2013; Torruella et al. 2015; Hehenberger et al. 2017), as they branch phylogenetically close to the metazoan radiation, being sister to Choanozoa (the Metazoa + Choanoflagellatea clade) (Shalchian-Tabrizi et al. 2008; Paps et al. 2013; Torruella et al. 2015; López-Escardó et al. 2019). Morphological (James-Clark 1868), ultrastructural (Laval 1971; Hibberd 1975), and phylogenetic inference (Cavalier-Smith 1993; Wainright et al. 1993; Snell et al. 2001; King 2004; Ruiz-Trillo et al. 2006) suggested a common evolutionary origin for Metazoa and Choanoflagellatea, which was confirmed by phylogenomic analyses (King et al. 2005; Steenkamp et al. 2006; Ruiz-Trillo et al. 2008). Phylogenomic studies also revealed the relationship between genera *Capsaspora* and *Ministeria* and their sister-clade relationship to Choanozoa (Shalchian-Tabrizi et al. 2008; Torruella et al. 2012; Hehenberger et al. 2017). Since then, the genomes and transcriptomes of filasterean species have been thoroughly investigated to comprehend the evolutionary processes that drove the inception of animal multicellularity (Suga et al. 2013; Torruella et al. 2015; Sebé-Pedrós et al. 2017; Hehenberger et al. 2017; Grau-Bove et al. 2017).

For almost 40 years, our knowledge of filasterean ultrastructure came from a single paper (Owczarzak et al. 1980), describing *C. owczarzaki*. Recently, the ultrastructures of *M. Vibrans* and *Pigoraptor* spp. have been investigated (Torruella et al. 2015; Mylnikov et al. 2019;

Tikhonenkov et al. 2020). Regarding the ecology and global distribution of the species within the Class, existing information is limited to the sampling locations of type species, and some feeding observations under culture conditions (Stibbs et al. 1979; Tong 1997; Hehenberger et al. 2017; Mylnikov et al. 2019; Tikhonenkov et al. 2020). Given the low number of species described, the influence of filastereans in the food web has been thought to be insignificant, at least in comparison to much bigger protistan clades or notorious pathogenic taxa. Recent environmental studies have suggested the relationship between an abundant clade of marine opisthokonts (MAOP-1) and Filasterea (del Campo et al. 2015; Hehenberger et al. 2017; Heger et al. 2018), challenging the idea of a small and scarce group. Excluding the facultative endosymbiont *C. owczarzaki,* all filastereans and choanoflagellates are free-living organisms, contrasting with the parasitic lifestyle of ichthyosporeans (mesomycetozoeans) (Mendoza et al. 2002; Glockling et al. 2013). The clade includes important pathogens of fish (Ragan et al. 1996; Pekkarinen & Lotman 2003; Andreou et al. 2011), amphibians (Broz & Privora 1952; Pereira et al. 2005; Rowley et al. 2013), birds, and mammals, including humans (Fredricks et al. 2000; Silva et al. 2005).

During a histopathological survey of invertebrates inhabiting the intertidal zone (Weymouth, UK), an unidentified protist was observed parasitizing two of the most common species of amphipods (*Echinogammarus* sp. and *Orchestia* sp.). Analysis by light microscopy of the structure and tissue tropism of the parasite did not allow a clear assignment of the organism to any of the pathogen groups commonly observed infecting amphipods or crustaceans. Similarly, examination of the ultrastructure by transmission electron microscopy (TEM) did not show any distinctive organelle suggesting taxonomic affiliation. Preliminary phylogenetic analyses of the 18S SSU rRNA strongly indicated that this lineage was a highly divergent novel genus within Holozoa. However, it did not consistently branch with any of the four established unicellular clades (Choanoflagellatea, Filasterea, Corallochytrea/Pluriformea, and Ichthyosporea/Mesomycetozoea). When a greater diversity of environmental holozoan sequences was included in the analyses the parasite branched with Filasterea as the earliest diverging branch. This phylogenetic position was substantiated by a phylogenomic analysis using a 96 protein-coding multi-gene tree built from a preliminary draft genome of the parasite, curated from the meta-genome constituting an infected amphipod sample. This study comprises a complete histopathological, ultrastructural, and phylogenetic analysis based on the complete 18S SSU rRNA of the novel parasite, described as *Txikispora philomaios* n. sp. n. g. We also present data on its prevalence, host range, biological cycle, and potential transmission routes. Additionally, we demonstrate novel filasterean diversity on the basis of sequences mined from

environmental sequencing datasets. The description of *T. philomaios* and its parasitic lifestyle adds to a growing understanding of filasterean diversity, ecology, and lifestyle traits.

2. MATERIALS AND METHODS

2.1. Sample collection

Amphipods belonging to genera *Orchestia*, *Echinogammarus*, *Gammarus*, and *Melita* were collected in the Tamar estuary (Torpoint, Cornwall), Camel estuary (Padstow, Cornwall), Dart estuary (Dittisham, Devon) and Newton's Cove (Weymouth, Dorset), all in southwest England, between 2016 and 2018 (Table1; Fig. 1). Individuals of *Echinogammarus* sp. and *Orchestia* sp. were sampled in the upper part of the intertidal zone, behind rocks and algae. Individuals of *Gammarus* sp. and *Melita* sp. were sampled in the lower part of the intertidal behind small stones and submerged algae. In addition to amphipods, other very abundant invertebrates sharing the same habitat in the upper part of the intertidal were also collected in Newton's Cove from May 2019 to September 2019 (Table 2). These organisms include *Capitella* sp. (Polychaeta; Annelida), *Procerodes* sp. (Turbellaria; Platyhelminthes), and harpacticoid copepods of the Ameiridae Family (Crustacea; Arthropoda), all individually selected using a stereomicroscope.

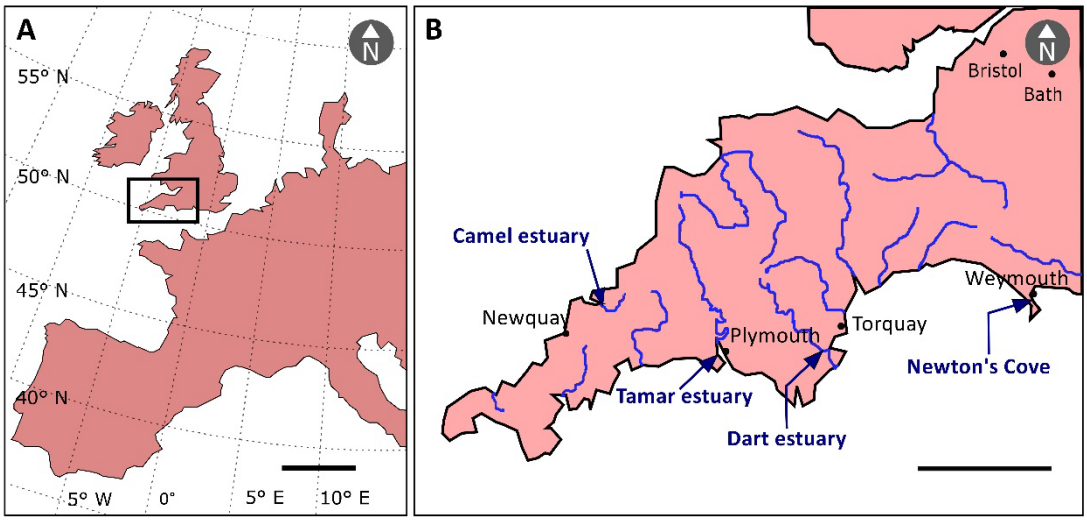


Figure. 1: Map showing the coastal locations in which amphipods of the genera *Echinogammarus*, *Orchestia*, *Melita* and *Gammarus* were collected. **A)** Western Europe, the black rectangle showing the area of UK sampled. **B)** Area contained within the black rectangle in (A). The blue lines show the rivers and estuaries; arrows indicate the sampling locations. Precise coordinates of the locations in Table 1.

Table 1: Amphipods collected by this study for full histopathological screening. Number of individuals belonging to different genera appear linked to the location and day of the sampling.

Sampling location	Location coordinates	Date	Number of amphipods sampled			
			Echinogammarus sp.	*Gammarus* sp.	*Melita* sp.	*Orchestia* sp.
Dart Estuary	50° 23' 21'' N 03° 35' 36'' W	19-Sep-16	64	10	10	31
		26-Apr-17	40	6	23	21
		13-Aug-18	41	0	0	0
Tamar estuary	50° 23' 25'' N 04° 13' 51'' W	20-Sep-16	84	0	4	28
		27-Apr-17	41	27	18	37
		14-Aug-18	48	0	0	34
		08-Nov-18	35	7	0	27
Camel Estuary	50° 32' 17'' N 04° 56' 05'' W	28-Apr-17	30	29	6	37
Newton's Cove (Weymouth)	50° 36' 17'' N 02° 27' 03'' W	20-Apr-16	50	0	0	0
		08-Jun-16	30	0	0	0
		13-Sep-16	38	30	13	32
		28-Oct-16	30	20	0	0
		25-Nov-16	40	0	0	0
		14-Dic-16	63	42	9	0
		17-Jan-17	40	30	6	0
		16-Feb-17	50	23	4	0
		16-Mar-17	54	0	0	6
		11-Apr-17	38	15	5	0
		04-May-17	51	0	0	0
		18-May-17	31	0	0	0
		15-Jun-17	12	10	0	25
		21-Jul-17	40	10	0	23
		16-Mar-18	55	12	0	10
		16-Apr-18	45	8	0	4
		11-May-18	31	0	0	0
		13-Jun-18	55	0	0	0

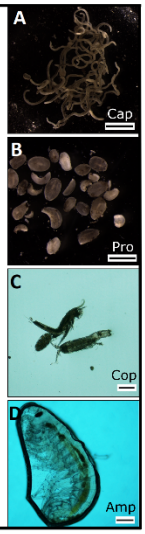
2.2. Histology and transmission electron microscopy

Amphipods were kept alive in bottles containing moist algae and dissected within 3-4 hours post collection. The head and two first thoracic segments were fixed in 100% molecular grade ethanol. The following proximate segments of the thorax of about 2 mm in size, were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for TEM. The remainder of the body, which included the last 4-5 segments of the pereon and the pleon, were fixed in Davidson's seawater fixative (Hopwood 1969) for 24 hours, and then transferred to 70% ethanol. Fresh smears were produced by cutting the distal part of the antennae or uropods before fixation; after a preliminary analysis, slides were left to air-dry. Once dry, slides were stained for 1 minute with Toluidine Blue (1%) and washed with distilled water before being cover-slipped.

For histology, Davidson's fixed samples were processed from ethanol to wax in a vacuum infiltration processor using established laboratory protocols (Stentiford et al. 2013). Tissue sections (2.5-3 µm) were cut on a Finnese® microtome, left to dry for 24 hours, mounted on VWR™ microscope slides, and stained with H&E (Bancroft and Cook 1994). Cover-slipped sections were examined for general histopathology by light microscopy (Nikon Eclipse E800). Digital images and measurements were obtained using the Lucia™ Screen Measurement software system (Nikon, UK).

Table 2: Sampling information for invertebrate species collected for PCR screening of *Txikispora philomaios* in Newton's Cove. The sampling date, the organism's genus/clade, and the number of individual organisms included in each batch. Between brackets in "**Organism**", stereomicroscopical images of the taxa (A, B, C, D). Between brackets in "**No. of Individuals**" the total number of individuals per PCR tube.

Sampling location	Date	Organism	Number of individuals
Newton's Cove, Weymouth, UK. (Upper intertidal) Coordinates: 50° 36' 17'' N 02° 27' 03'' W	21-may-19	*Capitella* sp. (A)	45 (3 tubes, 15 ind. each)
		Procerodes sp. (B)	60 (2 tubes, 30 ind. each)
		Echinogammarus sp. (D)	100 (4 tubes, 2 x 20 big 2 x 30 small)
		Copepoda (C)	300 (2 tubes, 150 ind. each)
	10-jul-19	*Capitella* sp.	90 (3 tubes, 30 ind. each)
		Procerodes sp.	90 (3 tubes, 30 ind. each)
		Echinogammarus sp.	100 (4 tubes, 2 x 20 big 2 x 30 small)
		Copepoda	400 (2 tubes, 200 ind. each)
	05-ago-19	*Capitella* sp.	120 (4 tubes, 30 ind. each)
		Procerodes sp.	120 (4 tubes, 30 ind. each)
		Echinogammarus sp.	100 (4 tubes, 2 x 20 big 2 x 30 small)
		Copepoda	100 (1 tube)
	28-sep-19	*Capitella* sp.	120 (4 tubes, 30 ind. each)
		Procerodes sp.	120 (4 tubes, 30 ind. each)
		Echinogammarus sp.	100 (4 tubes, 2 x 20 big 2 x 30 small)
		Copepoda	500 (2 tubes, 250 ind. each)



Specimens observed by light microscopy to be infectedwith *T. philomaios* (one *Echinogammarus* sp. and one *Orchestia* sp.), were selected for TEM analysis. Glutaraldehyde-fixed samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 hour in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Then, they were embedded in epoxy resin 812 (Agar Scientific pre-Mix Kit 812, Agar Scientific, UK) and polymerised overnight at 60 °C. Semi-thin sections (1 µm) were stained with 1% Toluidine Blue and analysed by light microscope, to identify target areas containing sufficient parasites. Ultrathin sections (70-90 nm) were framed on uncoated copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using a GATAN Erlangshen ES500W camera and Gatan Digital Micrograph™software.

2.3 DNA extraction, polymerase chain reaction, cloning and sequencing

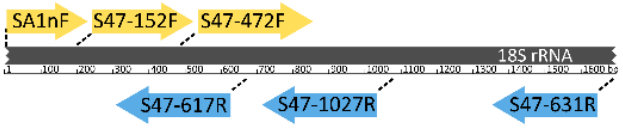
The head and anterior part of the thorax (preserved in 100% molecular grade ethanol) of 23 amphipods found to be infected via histology (pereon, pleon, and uropods fixed in Davidson's seawater fixative) were selected for DNA extraction. Infected tissues were disrupted and digested overnight (12 hours) using Fast Prep® Lysing Matrix tubes containing 0.2 mg (6 U) Proteinase K (Sigma-Aldrich®) diluted 1/40 in Lifton's Buffer (100 mM EDTA, 25 mM Tris-HCl, 1% (v/v) SDS, pH 7.6). Next, a 1/10 (v/v) of 5 M potassium acetate was added to each of the 23 tubes

containing digested sample, Proteinase K, and Lifton's buffer. The solution was mixed and incubated on ice for 1 hour. From here DNA was extracted using the phenol-chloroform method described in (Sambrook et al. 1989). The resulting pellet was diluted in 50 µl of molecular grade water and DNA concentration quantified using NanoDrop™ (Thermo Fisher Scientific). *T. philomaios*' 18S SSU rRNA (hereafter '18S') was amplified by PCR using primers targeting different overlapping regions (Table 3), and the following PCR conditions: A total reaction volume of 20 µl included 10 µl molecular water, 5 µL GoTaq® Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide, 40 pM of each primer, 0.5 U GoTaq® Polymerase (Promega), and 200 ng of the extracted DNA. The PCR cycling parameters for primer pair (SA1nF + 631R; Bass et al. 2012, and in-house design respectively; Table 3) included denaturation for 5 minutes at 95 °C, followed by 35 cycles alternating: 95 °C (30 s),57 °C(30 s), and 72 °C (90 s); before a final extension and incubation of the amplicons at 72 °C for 10 minutes. Same conditions were used for primer combinations (S47-152F + S47-617R and S47-472F + S47-1027R; Table 3) except for the annealing temperature, which was 67 °C (30 s). Amplicons were cleaned using 20% polyethylene glycol 8000 (Sigma-Aldrich®) followed by ethanol precipitation, and a-tailed to improve cloning efficiency before another PEG 8000 clean. Clone libraries were created using Strategene's cloning kit (Agilent Technologies, Santa Clara, CA, USA) as per manufacturer's protocol. Bacterial colonies were picked from LB/ampicillin plates and suspended in 20 µl PCR water and lysed at 95 °C for 5 minutes. Eight clones from each library were amplified with 1 µl lysed culture DNA and M13F/M13R primers (Invitrogen™ – Thermo Fisher Scientific) using the "mastermix" concentrations described previously, and the manufacturers program. PCR products were bead-cleaned and a total volume of 15 µl was mixed with 2 µl of the M13F forward primer, before being single-read Sanger sequenced (Eurofins®Genomics).

Table 3. List of primers designed for *Txikispora philomaios* amplification and universal primer SA1nF (*) from Bass et al. 2012. The melting temperature (Tm) and the sequence for each primer is specified. In the bottom, a diagram indicating position of attachment for each primer in the 18S ssu rRNA and direction of amplification.

Primers for *Txikispora philomaios* 18S ssu rRNA

Primer	Tm	Sequence (5' - 3')
SA1nF *	65.9°C	ACCTGGTTGATCCTGCCAGT
S47-152F	68.0°C	AGCTAATACATGCTGCAAAGCGG
S47-472F	73.2°C	TACCGGGCCTTCAAGGCACG
S47-617R	74.5°C	CGCTTTCACGCGACCATCACAAC
S47-1027R	71.6°C	ATACGGTGCCGAGAGCGTCAAA
S47-631R	62.7°C	CTCCCAAGACCTCACTAAATCAC



2.4 *In-situ* hybridization

Tissue sections (4 µm) from the individuals of interest were recovered from the 42 °C water bath (without Sta-On tissue-adhesive) using Polysine® Slides (Thermo Fisher Scientific) and left to dry for 24 hours. The forward S47-152F and reverse S47-617R primers were used to amplify part of the 18S extracted from an infected individual of *Orchestia* sp. DNA amplification and purification were carried out using the same concentrations and conditions explained in section (2.3). Purified DNA was digoxigenin(DIG)-labelled using same primers and PCR conditions above, but changing the concentration of reagents, say: 10 µl 5X Colorless GoTaq® Reaction Buffer, 5 µl $MgCl_2$ solution (Promega), 5 µl of PCR DIG labelling mix (Roche), 3 µl template DNA, 1 µl of forward and reverse primers, 0.5 µl of GoTaq Polymerase, and 24.5 µl molecular grade water. The control slide was produced amplifying the same 18S region using non-labelled standard DNTPs. Products generated via PCR were purified as described in previous section, total DNA quantified (NanoDrop 1000 Spectrophotometer® Thermo Scientific) and diluted to 1 ng/µl for a total volume of 50 µl.

Dry tissue sections were dewaxed and rehydrated: Clearene for 5 minutes (2 times), followed by 100% IDA (industrial denatured alcohol) for 5 minutes and 70% IDA another 5 minutes. Slides were rinsed in 0.1 M TRIS buffer (0.1 M TRIS base, 0.15 M NaCl, adjust the pH to 7.5 adding HCl) and placed in a humid chamber. Each slide was covered with 300 µl of 0.3% Triton-X diluted in 0.1 M TRIS buffer (pH 7.5) for 20 minutes and rinsed with 0.1M TRIS buffer (pH 7.5). Tissue was covered with Proteinase K diluted to 25 µg/ml in pre-warmed (37 °C) 0.1 M TRIS buffer (pH 7.5) and kept for 20 minutes at 37 °C within the humid chamber to prevent evaporation. Slides were washed in 70% IDA for 3 minutes and 100% IDA for another 3 minutes before rinsing them in SSC 2X for 1 minute while gently agitating (SSC 1X is 0.15 M sodium chloride and 0.015 M sodium citrate). Slides were kept in 0.1 M TRIS buffer (pH 7.5) until the *in-situ* hybridization frame seals (BIO-RAD) were glued to the slide around the sample. Then, the DIG-labelled probe and the non-labelled probe (control), both 50 µl in volume, were diluted by adding 50 µl of hybridization buffer and added to the cavity created by the gel frames in the slide, with the sample in the middle. After DNA denaturation at 94 °C for 6 minutes, slides were hybridized overnight (16 h) at 44 °C.

Samples were washed for 10 minutes with room temperature washing buffer (25 ml of SSC 20X, 6 M Urea, 2 mg/l BSA), before being washed twice with pre-heated (38 °C) washing buffer for 10 minutes each. Slides were rinsed with preheated (38 °C) SSC 1X for 5 minutes (2 times) and with 0.1 M TRIS buffer (pH 7.5) another 2 times. The blocking step was carried out

with a solution of 6% dried skimmed milk diluted in 0.1 M TRIS buffer (pH 7.5) for 1 h at room temperature and washed with 0.1 M TRIS buffer (pH 7.5) for 5 minutes, twice.

Slides were incubated with 1.5 U/ml of anti-DIG-AP Fab fragments (Roche) diluted in 0.1 M TRIS buffer (pH 7.5) for 1 h at room temperature in darkness. The excess of Anti-DIG-AP was removed by 4 successive washes in 0.1 M TRIS buffer (pH 7.5) for 10 minutes each. Slides were transferred to 0.1 M TRIS buffer (pH 9.5), which is (0.1 M TRIS base, 0.1 M NaCl, adjust pH to 9.5 adding HCl) for 2 minutes and then tissue was covered with NBT/BCIP stock solution (Roche) diluted in 0.1 M TRIS buffer (pH 9.5) at 20 µl/ml. Then incubated in darkness and room temperature until the first clear signs of blue staining appeared (about 30 minutes). Slides were washed in 0.1 M TRIS buffer (pH 9.5) for 1 minute twice and stained with 1% Bismark Brown for 6 minutes. Finally, slides were dehydrated by immersing them for 30 seconds in 70% IDA, 45 seconds in 100% IDA and 2 washes in clearene for 1 minute each. Slides were air dried for 30 minutes and permanently cover-slipped with DPX mounting medium (Sigma-Aldrich).

2.5 Sequence alignment and phylogenetic analysis

The PCR-amplified 18S rRNA was BlastN-searched (Zhang et al. 2000) against the GenBank nucleotide (nt) database. Holozoan 18S rDNA gene sequences, as well as sequences from those uncultured organisms showing highest similarity, were downloaded and aligned with the consensus 18S rDNA gene sequence from *T. philomaios* in MAFFT v.7 (Katoh et al. 2017) using the accurate option L-INS-i. The alignment was trimmed by TrimAL v.1.4.rev22 (Capella-Gutiérrez et al. 2009) using the (-gt 0.1) option, and manually curated in SeaView v.4 (Gouy et al. 2010). In turn, the best-fitting model (GTR + F + G) for the alignment was selected using ModelFinder (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE v.1.6.10 (Nguyen et al. 2015) and used to generate a ML tree in IQ-TREE. Branch support was obtained from 1,000 ultrafast bootstrap values (Minh et al 2013). A second maximum likelihood phylogenetic tree was constructed using RAxML v8.2.12 (Stamatakis 2014); support values calculated using 1,000 bootstrap replicates were mapped onto the tree with the highest likelihood value (evaluated under GTRGAMMA model). A Bayesian inference consensus tree was built using MrBayes v.3.2 (Ronquist et al. 2012) under default parameters except for the following: the number of substitution types was mixed; the model for among-site rate variation, Invgamma; the use of covarion-like model, activated. The MCMC parameters changed were: 5 million generations; sampling frequency set to every 1,000 generations; burnin-fraction value = 0.25; starting tree set to random, and all compatible groups in the consensus tree. A final consensus tree figure was created using FigTree v1.4.3 (Rambaut 2017) and based on the Bayesian topology.

A second 18S phylogenetic tree was constructed including environmental and unclassified sequences branching with or within Filasterea, by mining different databases. The 18S rDNA gene of *T. philomaios* was used as a bait to fish highly-similar sequences, by blastN searching against the following GenBank archives: nt, whole genome shotgun contigs (WGS), sequence read archive (SRA), and high throughput genomic sequence archive (HTGS). The same approach was followed for SILVA (www.arb-silva.de), ENA (www.ebi.ac.uk) and DDBJ (www.ddbj.nig.ac.jp) databases. All environmental sequences branching within Filasterea or sister to it in a preliminary tree were retained for subsequent analyses (Table 4), as were as a selection of highly divergent uncultured ichthyosporean and choanoflagellate sequences. Sequences belonging to uncultured organisms that branched robustly to existing species in Ichthyosporea, Choanoflagellatea, or Metazoa were excluded from the final alignment (the selected sequences were realigned). The alignment and subsequent phylogenetic analysis were constructed as described above.

2.6 Whole genome sequencing and phylogenomics

Phenol-Chloroform extracted DNA from heavily-infected amphipod tissues was purified using 20% polyethylene glycol 8000 (Sigma-Aldrich®) solution. The purified DNA was sent to the Centre for Genomic Research (University of Liverpool) in the volume and concentration by them specified.The DNA was shotgun-sequenced (Illumina Hiseq 2500) on two lanes, yielding 280,984,297 sequence reads. FASTQC (Andrews 2010) was used to assess the quality, length, GC content and number of paired and unpaired reads. Files containing paired forward (R1) reads from both lanes were merged with those containing paired reverse (R2) reads. Unpaired reads, which represented 2.34% of the total reads (6,427,037), were excluded from the downstream analysis. Forward and reverse reads were trimmed for adaptor sequences, contamination, and low-quality reads, using Trimmomatic v0.39 (Bolger et al. 2014), and the following parameters (Leading:3, Trailing:3, Minlen:36). Assembly of the resulting trimmed reads was carried out by two different widely used multi “K-mer” assemblers in metagenomic analysis, SPAdes (Bankevich et al. 2012) and MEGAHIT (Li et al. 2015).

The assembly generated by Megahit consisted of 2,529,706 contigs (N50 = 4,767 bp; L50 = 87,850 bp). Ultrafast metagenomic classification of the contigs carried out by Kraken v1.1.1 (Woods & Salzberg 2014) and subsequent visualization by Krona (Ondov et al. 2011) confirmed the presence of several protistan genomic assemblages in our sample. Contigs belonging to different lineages were separated into “bins”. Two commonly used programs were chosen to carry out this binning step; Concoct v1.1.0 (Alneberg et al. 2014) implemented the binning based

on coverage and sequence composition, while MetaBAT2 v2.12.1 (Kang et al. 2019) used tetranucleotide abundance and frequency.

The mapping of reads was performed with BWA-MEM mapping tool (Li 2013). Bins generated by Concoct (312 bins) and MetaBAT2 (35 bins) were analysed for completeness and contamination using CheckM (Parks et al. 2015) and BUSCO (Simão et al. 2015). CheckM was also used to identify the taxonomic position of each of the bins. The bin containing contigs that included *T. philomaios* 18S rRNA sequences contained 901 contigs comprising 23,600,000 bp. Program Anvi'o (Eren et al. 2015) was used to study the level of contamination indicated by CheckM and perform a supervised clean of unrelated contigs based on nucleotide rate and read depth. A fast alignment of the contigs remaining in the curated bin using Diamond v0.9.29.130 (Buchfink et al. 2015) allowed the detection of evident extraneous contigs. Theset were compared to the putative extraneous contigs selected by RefineM v0.1.1 (Parks et al. 2017) and removed in coincidental.

Table 4: (Next page) List of existing filastereans and uncultured organisms associated to this lineage according to our phylogenetic analysis (Fig. 10, 11). The sequence ID corresponds the name used in the phylogenetic trees, and it is linked to the ecosystem (sampling niche) and the geographic site (sampling location) from which the 18S ssu rRNA was collected. In the case of parasites and symbionts, susceptible hosts have been specified as the sampling niche. The list also includes the sequences' length, its identity (percentage) to *T. philomaios*' 18s, the database from which it was mined, and the reference to the authors who uploaded/published it.

TAXON	Species / Sequence ID	Sampling niche	Sampling location	Length	Identity with *T. philomaios*	Database	Reference
C. owczarzaki	AF349564.1	Symbiont (Mollusc)	Puerto Rico. (ATCC)	1797 bp	85.58%	GenBank (nr/nt)	Amaral-Zettler et al. 2001
C. owczarzaki	AF436888.1	Symbiont (Mollusc)	Brazil (ATCC)	1714 bp	85.47%	GenBank (nr/nt)	Amaral-Zettler et al. 2001
M. vibrans	AF271997.1	Coastal marine water	Cape Town, South Africa	1793 bp	83.59%	GenBank (nr/nt)	Cavalier-smith & Chao 2003
M. vibrans	AF271998.1	Coastal marine water	Southampton, UK	1795 bp	83.83%	GenBank (nr/nt)	Cavalier-smith & Chao 2003
P. chileana	MF190553.1	Sediments, Lake	Lago Blanca, Chile	1792 bp	87.48%	GenBank (nr/nt)	Hehenberger et al. 2017
P. vietnamica	MF190552.1	Sediments, Lake	Dak Lak, Vietnam	1794 bp	86.55%	GenBank (nr/nt)	Hehenberger et al. 2017
T. philomaios		Parasite (Crustacea)	Weymouth, UK	1679 bp	100.00%	GenBank (nr/nt)	This study
Unc. Filasterea	EU561669	Coastal marine water	Oyster Bay, South Africa	893 bp	87.10%	GenBank(wgs)	Not et al. 2008
Unc. Filasterea	FPLL01002905	Grassland	Aalborg, Denmark	1331 bp	85.95%	EBI/ENA (wgs)	Karst et al. 2016
Unc. Filasterea	FPLS01019718	Grassland	Aalborg, Denmark	1337 bp	86.02%	EBI/ENA (wgs)	Karst et al. 2016
Unc. Filasterea	GU825148.1	Marine (anoxic) water	Cariaco Basin, Venezuela	1067 bp	86.65%	GenBank (nr/nt)	Edgcomb et al. 2011
Unc. Filasterea	HQ870562.1	Marine (anoxic) water	Vancouver, Canada	840 bp	87.10%	GenBank (nr/nt)	Orsi et al. 2011
Unc. Filasterea	JQ223050.1	Marine (anoxic) water	Vancouver, Canada	1626 bp	86.50%	GenBank (nr/nt)	Unpublished
Unc. Filasterea	KT012912.1	Marine water	North Pacific (near Japan)	918 bp	90.17%	GenBank (nr/nt)	Unpublished
Unc. Filasterea	LN577465.1	Ant nest near river	Gamboa, Panama	730 bp	93.23%	EBI/ENA (nt)	Scott et al. 2014
Unc. Filasterea	LN580907.1	Ant nest near river	Gamboa, Panama	723 bp	91.90%	EBI/ENA (nt)	Scott et al. 2014
Unc. Filasterea	LN586076.1	Ant nest near river	Gamboa, Panama	725 bp	91.20%	EBI/ENA (nt)	Scott et al. 2014
Unc. Filasterea	LN586179.1	Ant nest near river	Gamboa, Panama	726 bp	91.25%	EBI/ENA (nt)	Scott et al. 2014
Unc. Filasterea	OBEP010137028	Algae and sediments, beach	Limfjorden, Denmark	1448 bp	90.44%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010162136	Algae and sediments, beach	Limfjorden, Denmark	1482 bp	84.93%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010275324	Ground water	Aalborg, Denmark	1530 bp	84.07%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010275456	Ground water	Aalborg, Denmark	1403 bp	83.80%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010275669	Ground water	Aalborg, Denmark	1235 bp	87.84%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010276073	Ground water	Aalborg, Denmark	1269 bp	85.07%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP010278239	Ground water	Aalborg, Denmark	1378 bp	87.74%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	OBEP011433235	Sediments, lake	Madum, Denmark	1179 bp	88.49%	EBI/ENA (wgs)	Karst et al. 2018
Unc. Filasterea	ORJL011316691	Ground water	South Glen Falls, NY, USA	1107 bp	88.34%	EBI/ENA (wgs)	Wilhelm et al. 2018

The downstream pipeline used 537 of the original 901 contigs from the bin putatively constituting *T. philomaios* genomic assembly. Program Augustus v3.3.3 (Stanke et al. 2006) was used to find protein-coding genes and their exon-intron structure in the contigs using default parameters and *Amphimedon queenslandica* as reference species. Augustus identified 4,327 protein-coding genes in the contigs (67% of the estimated genome length). This "draft genome" (sensu lato) of *T. philomaios* was analysed together with a selection of 255 single copy genes, from 38 specific holozoan species (Hehenberger et al. 2017) to find orthologs via Orthofinder v.2.3.3 (Emms & Kelly 2015). Concomitantly, the corresponding protein-coding genes from the following genomes downloaded in GenBank, predicted by Ausgutus, and connected via Orthofinder, were included in the analysis: *Dictyostelium purpureum* (GCA_000190715.1), *Acanthamoeba castellanii* (GCA_000826485.1), *Catenaria anguillulae* (GCA_002102555.1), *Mucor irregularis* (GCA_000587855.1), *Mortierella elongata* (GCA_001651415.1), *Batrachochytrium dendrobatidis* (GCA_000203795.1), *Rozella allomycis* (GCA_003614725.1). The resulting orthologous groups were aligned on MAFFT (using same parameters described in section 2.5); and trimmed on TrimAL (same parameters as in section 2.5). Phylogenetic trees for each gene were constructed using RAxML (LG+I+G; 100 bootstraps) to discard contamination and paralogy.

A selection of 96 aligned protein-coding genes from 46 different species, including *T. philomaios*, was concatenated using FASconCAT-G (Kück & Meusemann 2010). The phylogenetic analysis of the concatenated alignment was carried out using Maximum Likelihood (under RAxML and IQTREE) and Bayesian inference (MrBayes); parameters for RAxML (GTR + I + G; 1000 Bootstraps). IQTREE was used to select the best fitting model (LG + I + G) to generate a ML tree, constructed using the same program and 1,000 replicates (UF bootstrap support). MrBayes v.3.2 was used to build a Bayesian inference tree. The aminoacid-model was LG; the model for among-site rate variation, Invgamma; the use of covarion-like model was activated. The MCMC non-default parameters were: generation number = 250,000; sampling frequency set to every two generations; temp = 0.1; starting tree set to random; all groups compatible for consensus tree. The analysis stopped automatically after 170,000 generations, when it reached an average deviation < 0.01. A final consensus tree was created on FigTree v1.4.3 (Rambaut 2017) based on the Bayesian topology and including posterior probabilities, ML bootstrap, and ML ultrafast bootstrap.

A Hidden Markov Model (HMM) search of proteins involved in the flagellar formation and functioning was carried out against the annotated assemblies generated for *T. philomaios*, *P. vietnamica*, *P. chileana,* and the pluriformean *S. multiformis* (downloaded . Selected genes

were confirmed by BlastP searches against the GenBank protein database and construction of ML phylogenetic trees. Non-holozoan genes in *T. philomaios*' curated genomic assembly were discarded by phylogenetic trees of each protein/coding gene.

3 Results

3.1 . Clinical signs and prevalence

Two amphipod genera, *Echinogammarus* and *Orchestia*, were found infected by *T. philomaios*. The genera *Gammarus* (n = 279) and *Melita* (n = 101) were also investigated, but no signs of infection were observed histologically. However, the number of individuals examined was considerably lower (Table 1). Infection by *T. philomaios* was suggested macroscopically in heavily infected individuals by a yellowish and opaque integument (Fig. 2). The carapace thickened and lost rigidity (Fig. 2B), impeding to discern internal organs, especially the intestine, which was evident in young healthy individuals. Besides, gross examination of the most translucent appendages (antennae, uropods, and gills) using a stereomicroscope permitted detection of the parasite in haemolymph (Fig. 3). Infected individuals displayed lethargy, unresponsiveness to stimuli, and very reduced jumping ability in the case of the sand hopper (*Orchestia* sp.).

Fig. 2. Stereo-microscopical images of live *Echinogammarus* sp. amphipods collected in Newton's Cove. **A)** Uninfected individual. Antennae, pereopods and uropods (arrowheads), internal organs (arrow) **B)** Individual heavily infected by *Txikispora philomaios*. The tegument of the infected individual appears more opaque, the gut (arrow) is not evident, especially in the posterior fraction of the body (pleon). Scale bars = 100 µm for (A & B).

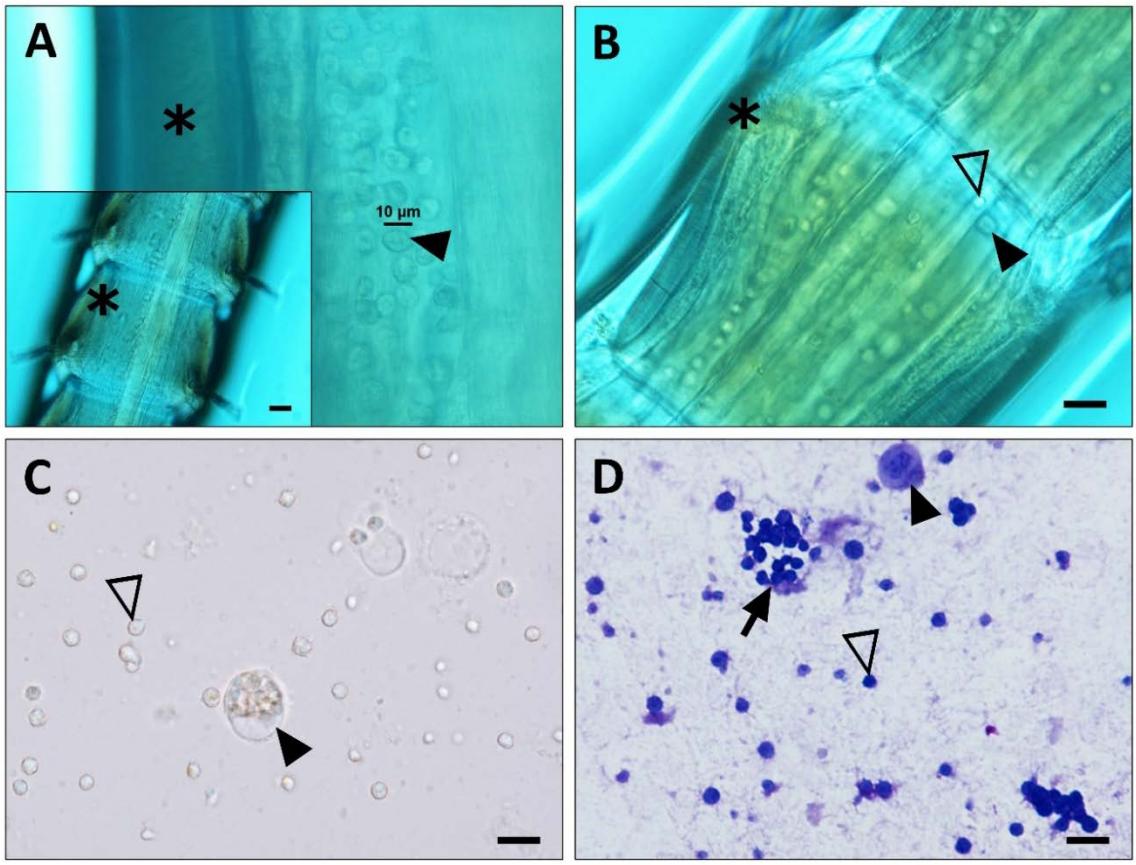


Fig. 3. Light microscopic images of antennae (**A**, **B**), and haemolymph (**C**, **D**) from healthy (A) and infected (B, C, D) amphipods of genus *Echinogammarus*. **A)** Stereo microscope image of the antennae (inset) of a healthy amphipod individual, showing (≈ 10 µm) haemocytes (arrowhead) flowing in the open circulatory system between the antennal gland and the tegument (asterisk). **B)** Cells of *Txikispora philomaios* (empty arrowhead) can be differentiated from haemocytes (filled arrowhead) by their smaller size and small nucleus. **C)** Composed microscope image of an unstained fresh preparation of the haemolymph showing *T. philomaios* cells free in the haemolymph (empty arrowhead) and within haemocytes (filled arrowhead). **D)**Toluidine Blue-stained preparation of haemolymph from an infected amphipod showing *T. philomaios* single cells (empty arrowhead), parasitic cells inside haemocytes (filled arrowhead), and parasite cells forming multicellular groups (arrow). Scale bars = 10 µm for (A, B,C, D), and 20 µm for inset in (A).

Discrimination between haemolymph cells (8-10 µm) and *T. philomaios* cells (2-4 µm) was possible on the basis of the cell diameter and nuclear size (Fig. 3A, 3B). Haemolymph smears (Fig. 3C) evidenced the difference between the spherical and peripheral nucleus of *T.philomaios* (~ 1 µm) and the central and irregular one in haemocytes (6-8 µm) (Fig. 3C). Additionally, fresh preparations allowed noticing the occurrence of up to 10 parasite cells inside host haemocytes. Toluidine Blue staining of the dry smears emphasised the structures, allowing the observation of cell aggregates (Fig. 3D). The occurrence of *T. philomaios* infection was consistent throughout the years of study (2016-2018) showing a distinct prevalence peak between late April and early June; at least for the regularly sampled *Echinogammarus* sp. population present in Newton's Cove.

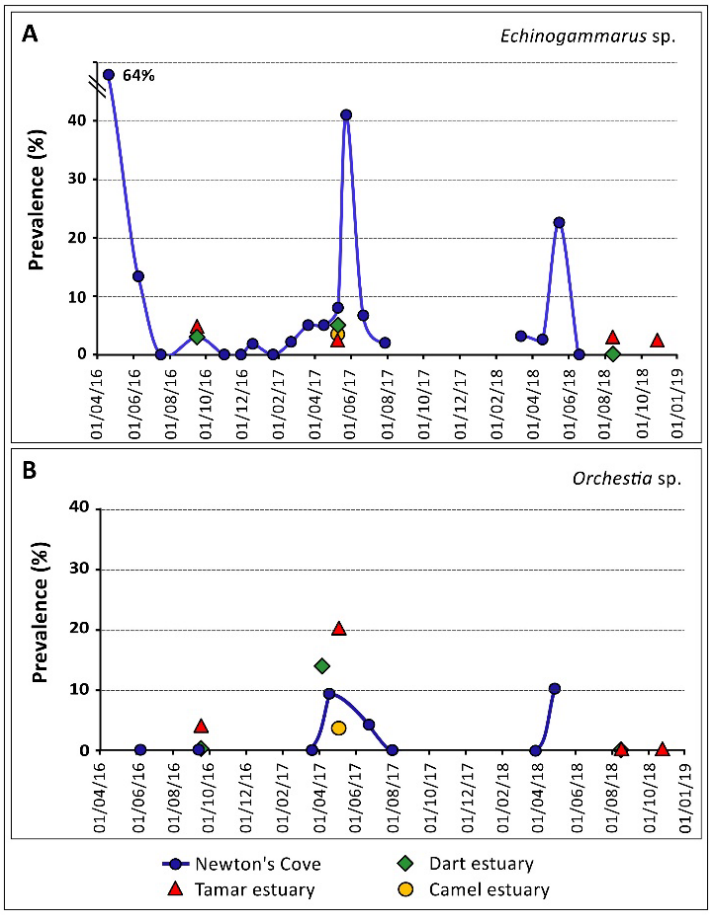


Fig. 4. Prevalence of *Txikispora philomaios* infection in *Echinogammarus* sp. **(A.)**, and *Orchestia* sp. **(B.)** from April 2016 to August 2018. Dates on the x-axis correspond to sampling information in (Table 1, 2). Y-axis:*T. philomaios* infection prevalence (%). Blue spheres refer to amphipods collected in Newton's Cove; red triangles = Tamar estuary; green diamonds = Dart estuary; yellow spheres= Camel estuary.

These outbreaks of *T. philomaios* infection were usually short-lived, usually lasting no more than three weeks. However, the prevalence of infection was high, varying between 24% (2018) and 64% (2016) in the coastal location of Weymouth. Although the limited data from the other sampling sites precluded direct comparison, the parasite was present in the Dart, Tamar, and Camel estuaries at low levels in both spring and autumn (Fig. 4A). *Orchestia* sp. was less frequently and abundantly sampled, but in Newton's Cove, infection also seemed to peak during May and early June (Fig. 4B). While in *Orchestia* sp. sampled in Newton's Cove the prevalence was lower (10%), the parasite was more frequently detected in the Dart and Tamar estuaries. The prevalence of infections in *Echinogammarus* sp. during the rest of the year (from June to early April) was low (1.9%, n = 1136), and infection was never systemic. The few parasitic cells observable during these months were almost exclusively associated with the testis.

3.2 Histopathology and ultrastructure

Cells of *T. philomaios* were virtually spherical (width = 1.94 ± 0.21 µm; length = 2.36 ± 0.23 µm; n = 50) when fixed in Davidson's seawater fixative, and 2.26 ± 0.34 µm by 2.60 ± 0.41 µm; n = 50) when preserved in glutaraldehyde. By light microscopy, a nucleus in the periphery of the cell was distinguished in a very translucent cytoplasm. Parasites were present in the haemolymph and frequently intra-cellularly within haemocytes (Fig. 5A). Infected haemocytes (containing up to 10 *T. philomaios* cells) were often necrotic, with a clear loss of cellular integrity; in contrast, the parasites inside them appeared to be intact. Aggregates of *T. philomaios* cells occurred free or within haemocytes, where similar sized stages were contained within a membrane. However, it was not possible to discern by light microscopy if aggregation was the result of single cells actively joining, or clusters of cells remaining together after the rupture of the haemocyte containing them. Proliferation of *T. philomaios* cells was associated with congestion of haemal sinuses of the tegumental gland and the connective tissue associated with the cuticular epithelium (Fig. 5B). In such systemic infections (with haemolymph, connective and tegument affected), *T. philomaios* was frequently observed infecting the hepatopancreas (Fig. 5C), and seldom in nervous tissues. In the hepatopancreas, the parasite was associated with structural damage with significant inflammation and granuloma formation, often encapsulating *T. philomaios* cells (Fig. 5C). The testis and ovary (Fig. 5D, 5E) also became infected, notably in early-stage infections. However, intracellular infections in oocytes/sperm were not observed.

At the TEM, *T. philomaios* was found more often as single cells, but also forming clusters containing 3 - 4 cells (Fig. 6A, 6B). Single cells, often coated by a cell wall, contained a pale staining nucleus with a peripheral compact nucleolus, small mitochondria with lamellar cristae and lipoid structures of varying electron-density (Fig. 6A). These lipoid inclusions displayed morphologic plasticity and variable staining characteristics between *T. philomaios* cells of different size (Fig. 6C, 6D). Electron-lucent granules appeared integrated within the cytoplasm, while darker granules were often membrane bound and associated to evaginations of the cell wall (Fig. 6G, 6H). The multi-layered cell wall varied in thickness (Fig. 6G, 6H, 6I) and in approximately 30% of the cells examined, appeared detached from the plasma membrane (Fig. 6A, 6G). In few cases, a matrix was observable between cell wall and the detached plasma membrane (Fig. 6C). A multicellular stage of *T. philomaios* was also frequently prominent (Fig. 6B); tri-cellular in appearance a hidden fourth cell was occasionally observed (Fig. 7D). In several multicellular clusters (Fig. 6B, 6E, 6F) the cells were indistinguishable from the unicellular stages present in the haemolymph (Fig. 6A, 6C, 6D).

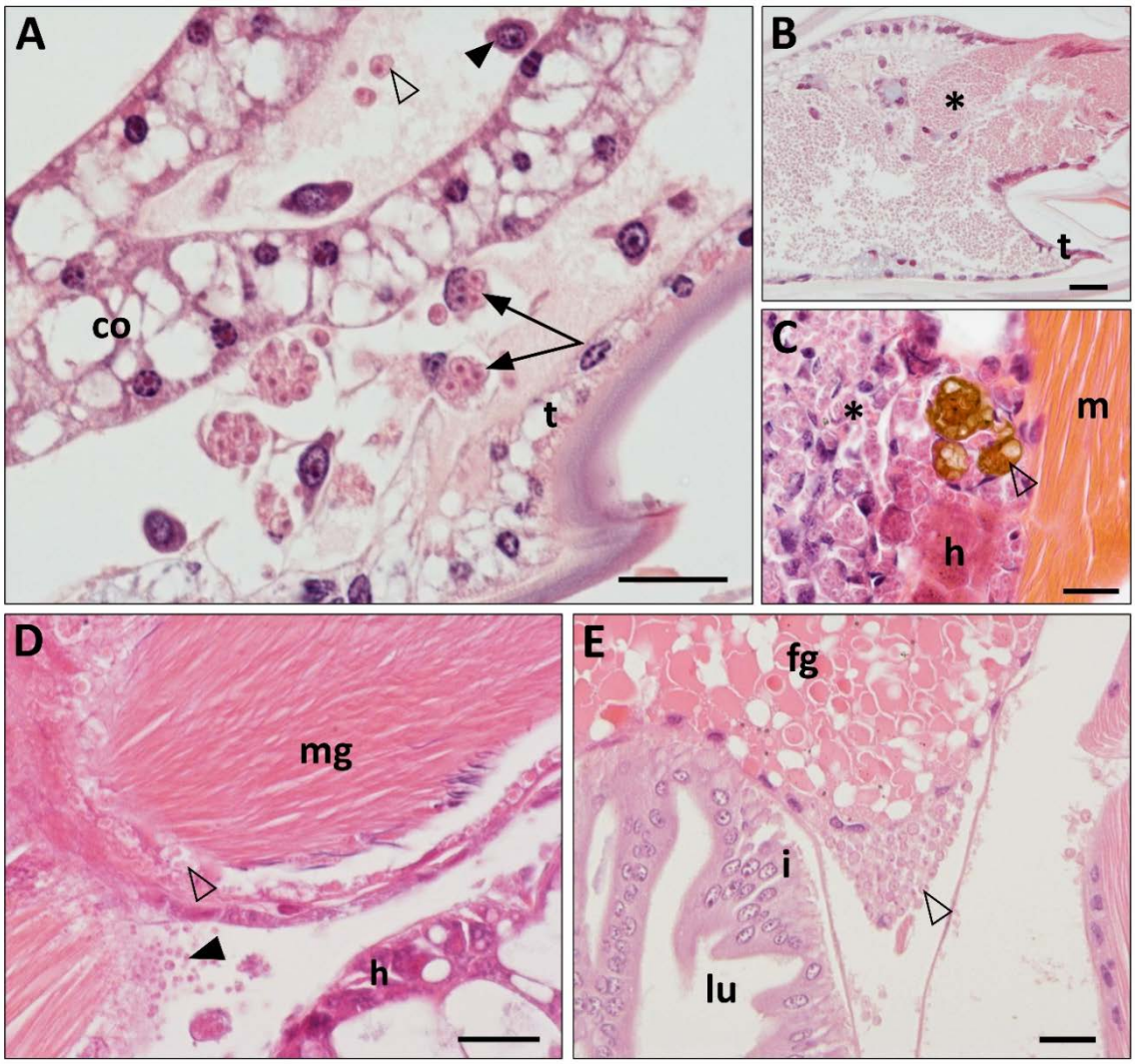


Fig. 5. Histological appearance of *Txikispora philomaios* infecting different tissues in *Orchestia* sp. **A)** Parasite cells were observed free in the haemolymph (empty arrowhead) and inside haemocytes (arrows). Non-infected haemocytes (filled arrowhead), tegument (t) and connective tissue (co), in the pereopods of the amphipod. **B)** Masses of parasitic cells (*) in the haemolymph and tegumental gland (t) associated with the cuticle of the carapace. **C)** Parasite cells (*) infiltrating the hepatopancreas (h). Granulomas and melanization (empty arrowhead) and muscle fibres (m). **D)** *T. philomaios* cells infiltrated between muscle fibres (filled arrowhead) and inner connective layers (empty arrowhead) of male gonads (mg). **E)** Disrupted female gonadal tissue (fg) associated to parasitic cells (empty arrowhead). Unaffected intestine (i) and its lumen (lu). Scale bars = 20 µm for (A,B,C,D,E).

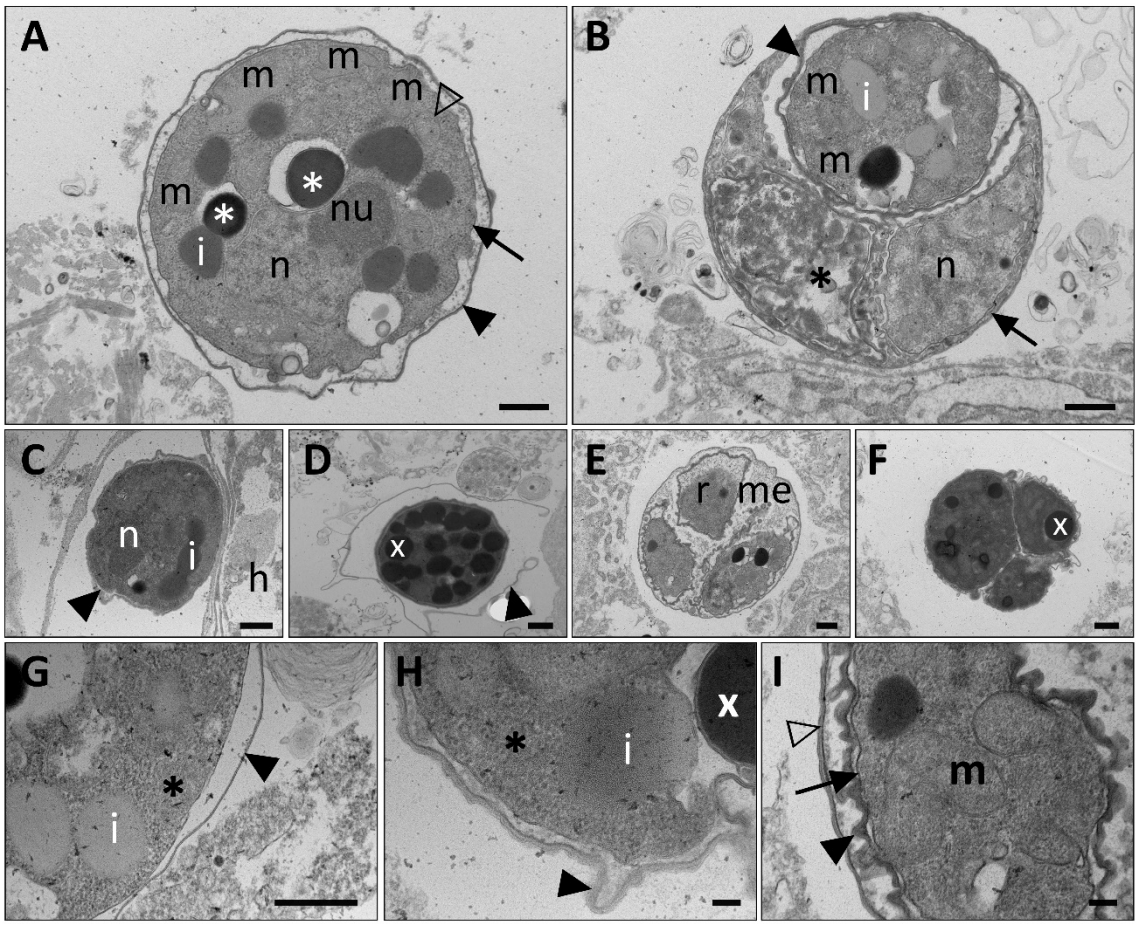


Fig. 6. Transmission Electron Microscope (TEM) micrographs of *Txikispora philomaios* cells infecting *Orchestia* sp. **A)** Unicellular stages of the parasite show a single amorphous nucleus (n) with a peripheral nucleolus (nu), peripheric mitochondria (m) electron-dense lipidic vesicles (*), and electron-lucent vesicles (i). The cell wall (filled arrowhead) appears detached from the plasma membrane (arrow). **B)** Dividing form of the parasite, with outer cell wall (arrow) and walled inner cells (filled arrowhead). One of the inner cells appears necrotic (*). **C)** Unicellular stage attached to host cell (h); amorphous material between wall and plasma membrane (filled arrowhead); (i) electron-lucent vesicles (reserve material). **D)** Unicellular stage full of electron-dense vesicles (x) with disrupted cell wall around (filled arrowhead). **E)** Dividing form, with inner cells (r) partially sharing the same matrixial material (me) with the outer walled cell. **F)** Electron-dense tricellular stage still within an indistinct walled outer cell. **G)** Detail of the thin wall (filled arrowhead) of a unicellular parasite cell inside a host haemocyte. Electron-lucent vesicles (i) and granular cytoplasm (*). **H)** Detail of a unicellular parasite cell with a thickening and evaginating cell wall (arrowhead). **I)** Detail of outer (empty arrowhead) and inner (filled arrowhead) cell walls, plasma membrane (arrow), and mitochondria (m). Scale bars = 500 nm for (A, B, C, D, E, F, G) and 100 nm for (H, I).

Occasionally, one or more individual cells contained within the walled parent cell were necrotic (Fig. 6B). Numerous peripheral mitochondria were observed in cells with a thickened wall (Fig. 6A, 6I). The thickening of the electron-dense wall of the inner cells was concurrent with a diminishing wall of the receptacle (Fig. 6D, 6F). The presence of unicellular and dividing forms of *T. philomaios* inside host haemocytes and tegumental gland hinted by light microscopy was corroborated by TEM analysis (Fig. 7A, 7B, 7C). Parasite cells appeared healthy in contrast to the

compromised integrity of the infected host cell (Fig. 7C). The multicellular form appeared more often within haemocytes (Fig. 7A, 7B), while unicellular stages were more commonly observed free in the haemolymph or inside cells of the host tegument (Fig. 7C).

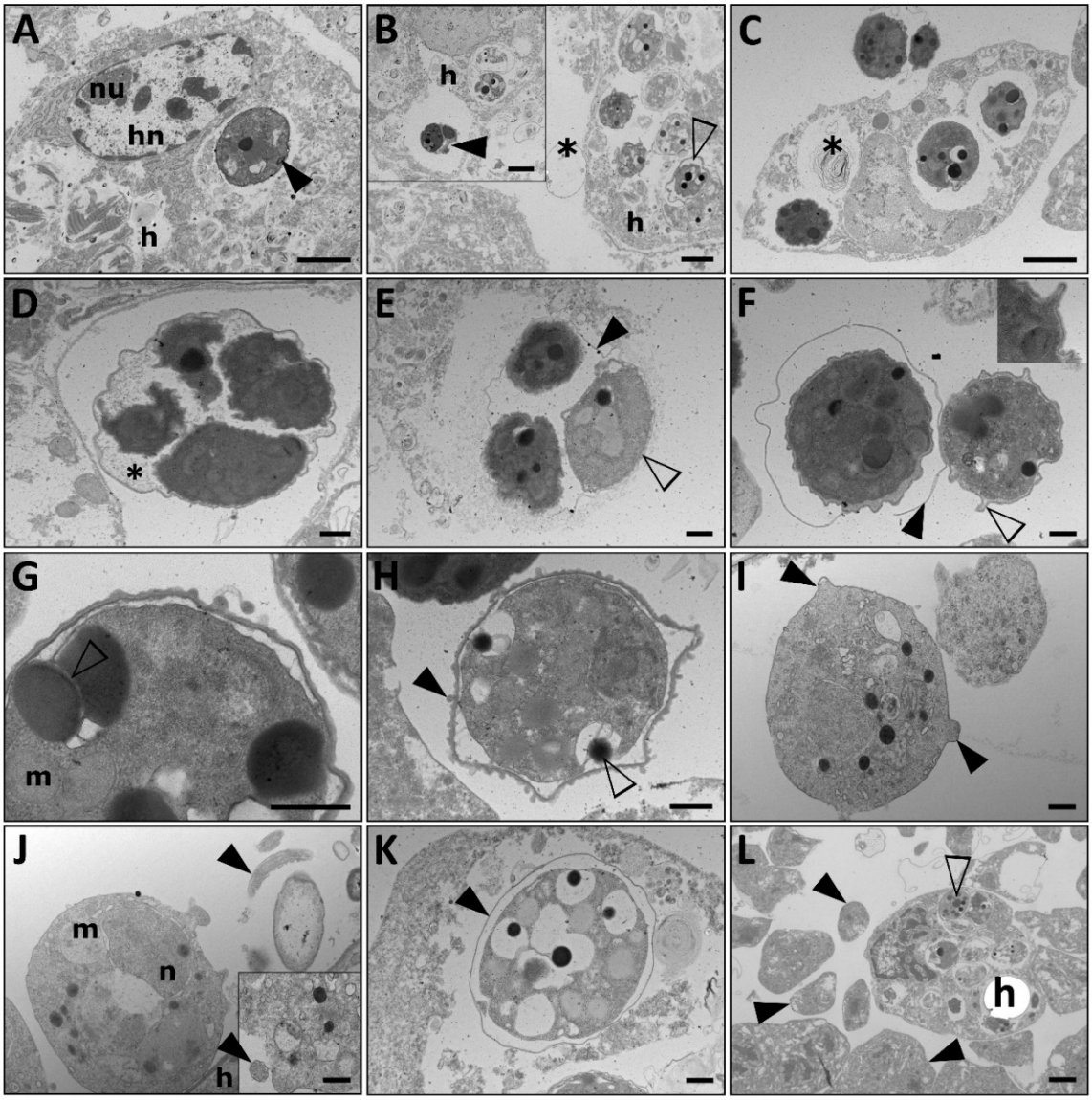


Fig. 7. Transmission Electron Microscope (TEM) micrographs of *Txikispora philomaios* cells infecting *Orchestia* sp. **A)** Intracellular stage of *T. philomaios* with a fine and closely attached cell wall (filled arrowhead). The host (h), its nucleus (hn), and nucleolus (nu) are shown. **B)** Five unicellular and a single multicellular stage (empty arrowhead) inside a host haemocyte (h), with a presumed parasite cell wall (*) attached to it. The inset shows the presence of a more electron-dense dividing form of *T. philomaios* (filled arrowhead) inside a host cell (h). **C)** Necrotic haemocyte containing three intact *T. philomaios* cells, with one vacuole containing a necrotic *T. philomaios* cell (*). The infected host cell is unable to maintain its normal structure, also true for its nucleus (hn). **D)** Divisional stage of *T. philomaios*. Four electron-dense daughter cells increase in size inside the wall of the parent cell, which still contains an evident cytoplasmatic matrix (*). **E)** Three daughter cells inside a parent cell without matrix and a very reduced cell wall (arrowhead). One of the daughter cells is more translucent (empty arrowhead) than its sister cyst-like cells. **F)** Two unicellular stages, one of them with an open thin wall (filled arrowhead) similar to the one marked with an asterisk in figure 7B. The other with short projections of the outer cell wall (empty

arrowhead). (Fig. 7-continuation). Detail of the inner structure of the projection in the inset. **G)** Detail of two electron-dense vesicles surrounded by a double lipidic membrane (empty arrowhead) in the immediate periphery of the cell. Mitochondria (m). **H)** Unicellular stage showing detachment of the outer cell-wall. The wall presents several subtle evaginations (filled arrowhead). An electron-dense vesicle (empty arrowhead) isexcreted to the space between plasma membrane and cell wall. **I)** Surface projections on a free *T. philomaios* cell (arrowheads). **J)** Parasite cell with mitochondria (m) and nucleus (n) in contact with a host cell (h). At least two flagellar structures (black arrows) have been observed flanking *T. philomaios* cells **K)** Intracellular stage of *T. philomaios* inside a host haemocyte with a thin detached wall (filled arrowhead) **L.** Coinfection of *T. philomaios* (empty arrowhead) and the ascetosporean parasite *Haplosporidium orchestiae* (filled arrowheads) in *Orchestia* sp. Only developing *T. philomaios* cells (empty arrowhead) are visible inside host haemocytes (h). Scale bars = 2 µm for (A, B, C, L), and 500 nm for (D, E, F, G, H, I, J, K). Inset in (B) is 2 µm; inset in (F) is 100 nm.

The majority of *T. philomaios* cells examined corresponded to one of the two main cell cycle stages described above. The occasional occurrence of intermediate forms and structures (Fig. 7E, 7F) suggested how unicellular cells were released from multicellular stages. The wall of the receptacle became reduced until it fractured, allowing dispersal of the walled inner cells. Just before being released, or immediately after (Fig. 7E, 7F), some of the released cells became less electron-dense, with a fine matrix between wall and plasma membrane. In later stages, the cell wall thickened and separated from the plasma membrane, possibly aided by co-occurring cellular projections (Fig. 7F). At this stage, some of the electron-dense lipid vesicles (Fig. 7G, 7H), seemingly enclosed by a double membrane, were absorbed, or excreted. Occurrence of non-walled unicellular forms of *T. philomaios* constituted the only stages in which the presence of microvilli (Fig. 7I) and maybe a flagellum (Fig. 7J) were noticeable. Inside haemocytes non-walled parasitic cells were loosely enclosed by a membrane of unknown origin (Fig. 7K). Co-infection of *T. philomaios* with *Haplosporidium* sp. (Urrutia et al. 2019) was not uncommon (Fig. 7L), but only *T. philomaios* cells were observed inside haemocytes.

The *In-situ* hybridization confirmed that the ultrastructure and histopathology of the amphipod infecting microeukaryotes matched with the 18S identified as *T. philomaios* (Fig. 8). The size and distribution of the DIG-NTB stained structures coincided with their immediate histological H&E stained sections. Round blue stains (2-4 µm) appeared concentrated in tegument, connective tissue, (Fig. 8A, 8B), gills, haemolymph (Fig. 8C, 8D), and inside haemocytes (Fig. 8E, 8F).

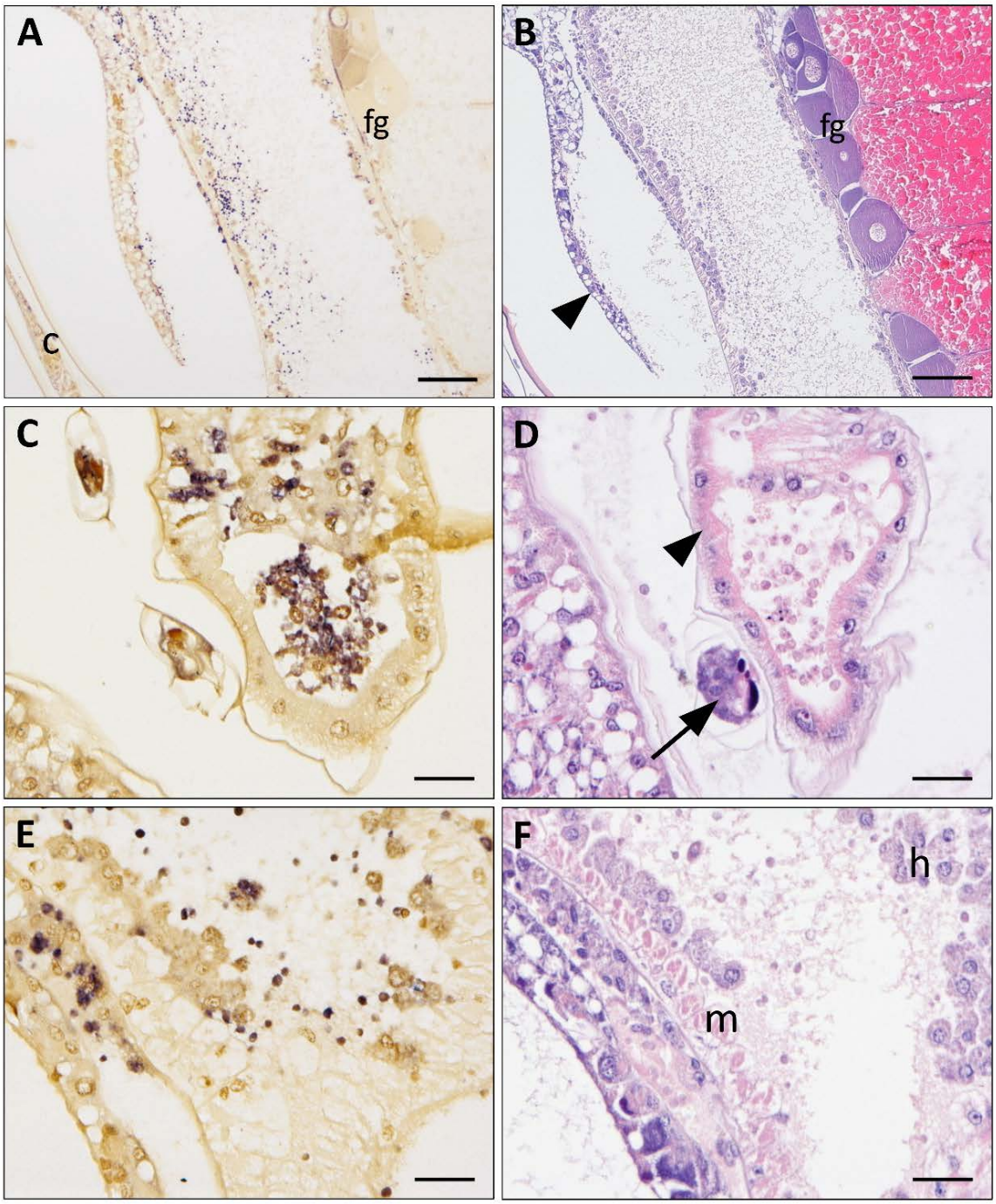


Fig. 8. Histological sections of *Orchestia* sp. tissues following *In-Situ* Hybridization (ISH) using a DIG-labelled probe (A,C, E) and the respective consecutive histologicalH&E-stained section obtained from the same host (B,D, F). **A** & **B)** *Txikispora philomaios* cells can be observed infecting the tegument (filled arrowhead) the cuticle (c) and haemocytes present in the cardiac tissues. Female gonads (fg) appear uninfected in this individual. **C** & **D)** Infected gill cells (arrowhead) usually have ciliates attached (arrow), which are not infected in this occasion. Haemolymph circulating through the gills is heavily infected with *T. philomaios* cells. **E** & **F.** Uninfected muscle (m) forming the cardiac tissue, pumps infected haemocytes (h) to other tissues. Scale bars = 100 µm for (A, B) and 25 µm for (C, D, E, F).

3.3 Life cycle and potential vectors

The occurrence of a multicellular stage provided strong evidence that *T. philomaios* was proliferating inside amphipod hosts. Two different amphipod genera were found to be susceptible to infection by *T. philomaios*, raising questions about host specificity. Therefore, some common invertebrates cohabiting with *Echinogammarus* sp. and *Orchestia* sp. were analysed histologically and by PCR. In Newton's Cove, co-occurring polychaetes of genus *Capitella*, the turbellarian *Procerodes* sp., and harpacticoid copepods were sampled (Table 2). While evident systemic *T. philomaios* infection in amphipods is limited to late April and May, we recognized the possibility that the parasite might be present in other hosts during a different time of the year. Thus, abundantly co-occurring invertebrates were sampled during May, June, July, August, and September. No clear evidence of *T. philomaios* cells was observed in the histopathological survey of *Procerodes* sp., *Capitella* sp. or harpacticoid copepods. However, PCR analysis carried out using sets of individuals representing these taxa indicated the presence of DNA of *T. philomaios* in a single sample comprising *Procerodes* individuals, collected during May 2019 (Fig. 9).

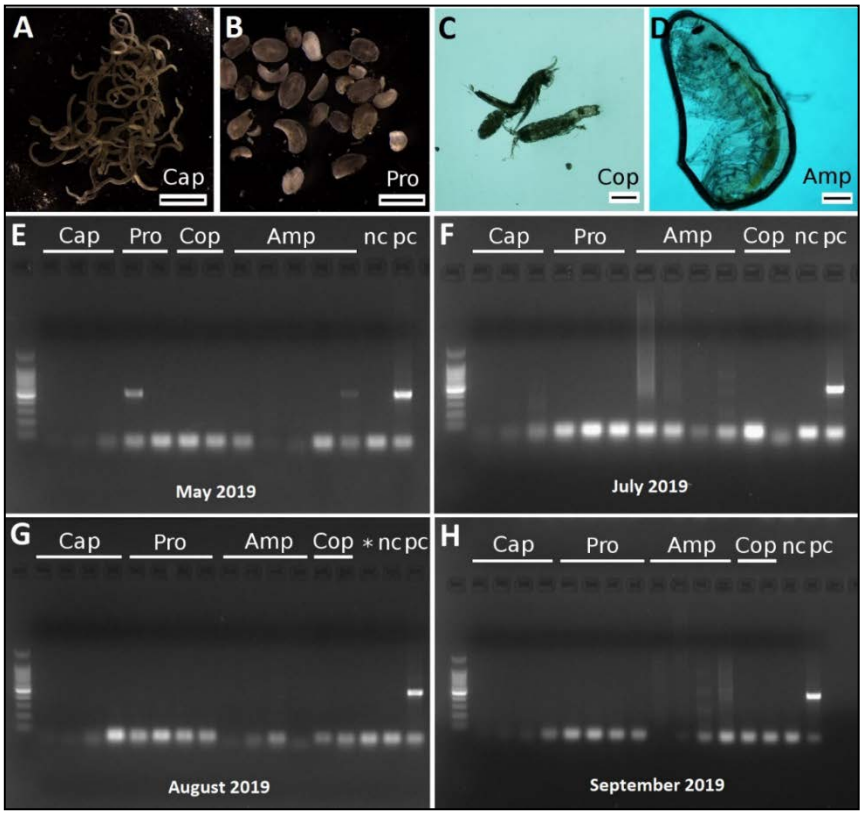


Figure 9: Analysis by gel electrophoresis of PCR amplified 18s rRNA fragments using *T. philomaios* specific primers on batches of individuals pertaining to the four organisms shown in the upper part of the figure. The organisms were collected from the upper part of the intertidal in Newton's Cove as specified in Table 2, and are represented as: **(A)** *Capitella* sp. **(B)** *Procerodes* sp. **(C)** harpacticoid copepods. **(D)** *Echinogammarus* sp. The results for the PCR runs shown by gel electrophoresis for the different months of samplings **(E, F, G, H)**. Cap = *Capitella* sp., Pro = *Procerodes* sp. Amp = *Echinogammarus* sp. Cop = harpacticoid copepods; nc = negative control. pc = positive control from a histologically determined *T. philomaios* infection. The steps of the ladder shown in the left of the gels are 100 bp. The asterisk in (G) belongs to some nematodes collected, but the number and genus was not determined, neither explained in materials and methods.

3.4 Phylogenetic analyses

Initially, a partial SSU sequence (ca. 705 bp long, including variable regions V5, V7, V8, and partial V9) was coincidentally amplified by haplosporidian-specific primers (Hartikainen et al 2014) from an *Echinogammarus* sp. sample later shown to be infected by *T. philomaios*. The top BlastN match for this sequence was the ichthyosporean *Dermocystidium salmonis* (91.5% similarity; 92% coverage; e-value = 0). Phylogenetic analysis of this 705 bp sequence (not shown) placed *T. philomaios* within clade Holozoa, with low branch support for any particular position, but often grouping with Ichthyosporea or Filasterea.A longer, equivalent 18S region of 1679 bp generated from an infected *Echinogammarus sp*. individual resulted in a BlastN match of 87.90% similarity (99% coverage) to the free living filasterean *Pigoraptor chileana*. Phylogenetic analysis of the 1679 bp region (Fig. 9) was consistent with that using the shorter fragment, and robustly placed *T. philomaios* as a holozoan, but very weakly branching as the earliest diverging lineage in Holozoa.

Several databases were mined for environmental sequences (process specified in section 2.5) related to *T. philomaios* (Table 4). The resulting phylogenetic tree (Fig. 10) showed some interesting differences when compared to the tree without environmental sequences (Fig. 10). In particular, in (Fig. 11) *T. philomaios* branched within Filasterea, in a clade mostly comprising environmental sequences, but also *Ministeria*. The filasterean clade was more strongly supported with the inclusion of the environmental sequences, with supports of (0.98, 21, 72; posterior probability, ML bootstrap, and ML ultrafast bootstrap, respectively) compared to (0.9, -, -) in (Fig. 11). The metazoan, choanoflagellate, and fungal clades were again fully/strongly supported, although the ML bootstrap support for the ichthyosporean clade was lower: 1, 34, 68 in (Fig. 10) to 0.99, 73, 82 in (Fig. 11). The phylogenetic position of the two pluriformean species as basal to choanoflagellates was maintained, but the support for *C. limacisporum* in that position increased from (0.32, -, 46) to (0.91, 19, 64).

The filasterean clade in (Fig. 11) was moderately well supported by Bayesian Inference (0.98, 21, 72) but contained a large proportion of partial environmental sequences yielding disparity between ML methods. *Txikispora* was robustly placed as sister to (Metagenome seq. OBEP010137028) sampled from sandy/muddy sediments associated with algae in Ulvedybet in Limfjorden (northern Denmark) (Karst et al. 2018). These, together with *Ministeria*, formed a clade with other environmental sequences from fresh groundwater systems in Denmark (OBEP010275669, OBEP010278239, OBEP010275324, OBEP010275456, OBEP010276073) and New York State (ORJL011316691) (Karst et al. 2018; Wilhelm et al. 2018), with the exception of OBEP010162136, which also came from the coastal location in Limfjorden (Karst et al. 2018).

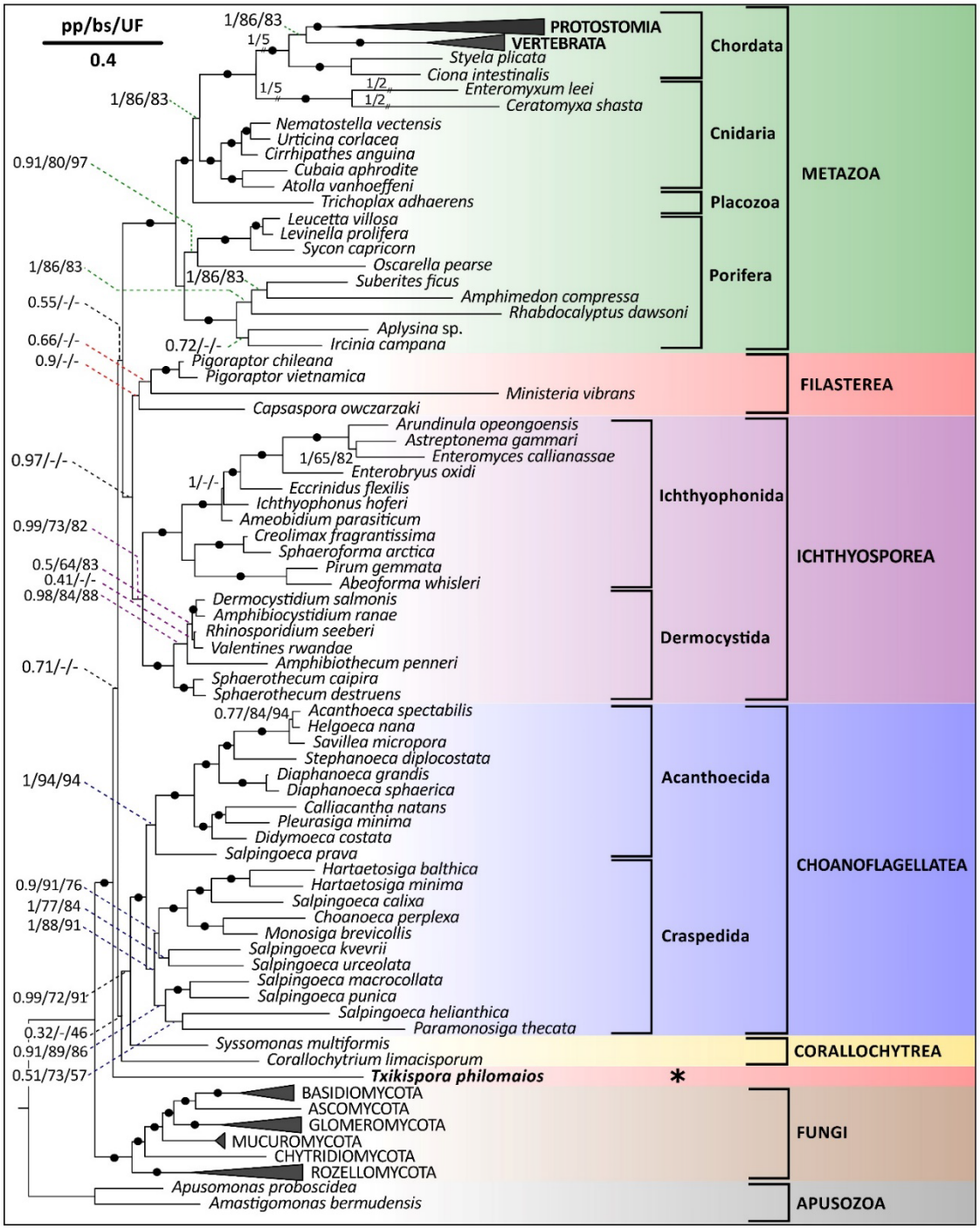


Fig. 10. Bayesian phylogenetic analysis of 18S and 28S rRNA genes places the novel amphipod parasite *Txikispora philomaios* (1679 bp) within Holozoa. The alignment included the 1679 bp 18S rRNA gene sequence of *T. philomaios* and the 18S of the rest of species (28S sequences were included where available in Genbank). The tree includes a selection of the main opisthokont groups and unicellular holozoan lineages. Branch support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum likelihood bootstrap support (bs) generated using RAxML with 1,000 replicates, and ML ultrafast 1,000 replicates bootstrap support (UF) from IQ-TREE, respectively. Branches with values (> 0.95 pp, > 95% bs, > 95% UF) are represented by a black dot on the branch. Species belonging to clades Protostomia, Vertebrata, Basydiomycota, Glomeromycota, Mucuromycota, Chytridiomycota, and Rozellida were collapsed.

The other characterised filasterean taxa, *Capsaspora* and *Pigoraptor*, grouped separately within the filasterean clade, and potentially more closely to each other than to *Ministeria* and *Txikispora* (Fig. 11)

Several environmental sequences branched close to *Capsaspora* and *Pigoraptor*. Metagenomic sequence OBEP01433235, collected from the sediments in a freshwater lake (Denmark) was very closely related to *Pigoraptor.* Additionally, two almost identical sequences (FPLL01002905 and FPLS01019718) collected from soil samples in Denmark (Karst et al. 2016) were robustly sister to *C. owczarzaki* (100, 100, 100).Two further clades of environmental sequences branched within the filasterean clade as shown on Fig. 10. One was an abundant group of uncultured marine organisms named “MAOP1 - Marine Opisthokonts”, which was weakly sister to *Pigoraptor* in Hehenberger et al. (2017). The other was a clade formed by short sequences (indicated on Fig. 11 as LN******) collected from a subterranean colony of ants adjacent to Chagres river, Panama (Scott et al. 2010).

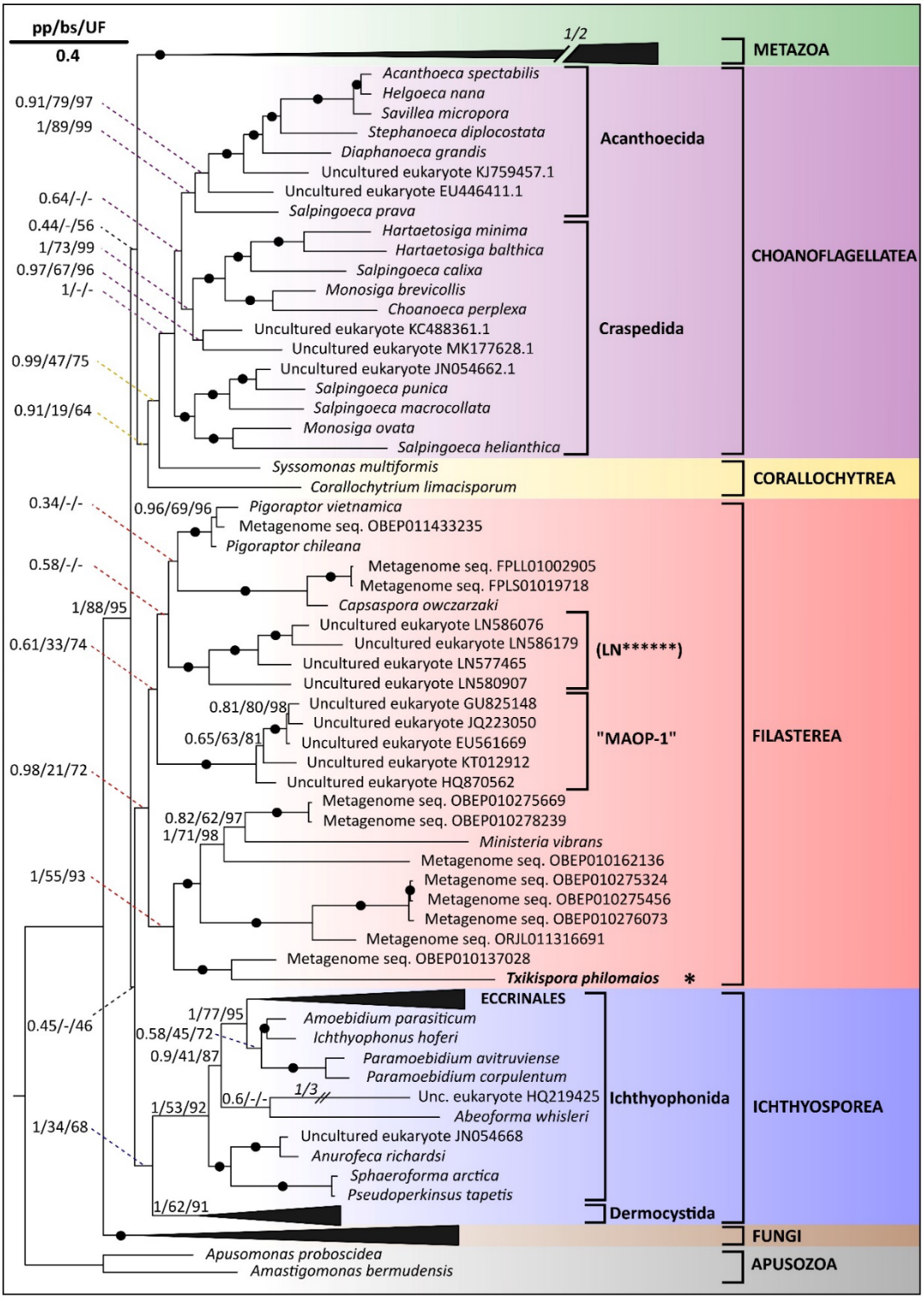


Fig. 11. Bayesian phylogenetic analysis of 18S and 28S rRNA genes, including environmental sequences, places the novel amphipod parasite *Txikispora philomaios* (1679 bp) within Filasterea. 28S sequences were included where available in Genbank.Branch support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on MrBayes, maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap support (UF) from IQ-TREE, respectively. Branches with values (> 0.95 pp, > 95% bs, > 95% UF) are represented by a black dot on the branch. Species belonging to Metazoa and Fungi were collapsed, as were Eccrinales and Dermocystida (Ichthyosporea). Environmental sequences areindicated by their GenBank accession numbers.

3.5 Phylogenomic and genome-based structural analyses

The phylogenetic analysis based on the 18S showed that *T. philomaios* groups within Holozoa, with full posterior probability and ML bootstrap support. While extensive phylogenetic trees including key environmental sequences indicated that the parasite was the earliest diverging branch in the filasterean clade, the moderate ML support did not allow us to confirm this phylogenetic position within the Class. Phylogenetic trees using concatenations of multiple genes have shown to provide better resolution for the phylogenetic positioning of many long-branching holozoans. The phylogenomic multigene analysis conducted using a selection of 96 protein-coding genes selected from preliminary draft genome of *T. philomaios* (≈ 6,500 genes) confirmed the phylogenetic position of the parasite as the earliest-diverging branch among filastereans.

Compared to the 18S trees (Fig. 10, 11), the number of taxa was significantly lower in the multigene tree (Fig. 12). This is explained by a lower number of available relevant non-metazoan genomes available for Holozoa, plus an exponential increase in the computational effort needed to deal with concatenated protein sequences. This resulted in a more strongly supported tree, particularly evident for the relationships between higher clades. The high-rank clades Choanozoa (Metazoa + Choanoflagellatea) and Filozoa (Metazoa + Choanoflagellatea + Filasterea) were fully supported (100, 100, 100). Pluriformea (*S. multiformis* + *C. limacisporum*) appeared as basal to Filozoa (100, 100, 100), and Ichthyosporea remained the most basal clade within Holozoa. The Bayesian posterior probability was virtually absolute for every bipartition in the tree and only few highly divergent clades within Fungi and Metazoa appeared to have incomplete ML bootstrap support. Such were the cases of relationship between Mucuromycota and Chytridiomycota (1, 58, 75), or vertebrates and the hemichordate *Saccoglossus kowalevskii* (0.98, 57, 69). Regarding *T. philomaios* the phylogenomic tree showed the parasite to be basal within class Filasterea, with strong Bayesian and moderate-high significance for the ML analysis. (1, 74, 96). Concomitantly to an extensive phylogenomic analysis, the draft genome of *T. philomaios* allowed the identification of gene orthologs related to multicellular aggregation and the formation of relevant phenotypical structures, including the flagellum. The pseudopods and flagellum-like structures observed on fresh haemolymph smears by light-microscopy were suggested but not demonstrated by ultrastructural analysis by TEM. However, the genomic analysis showed that all genes presumed to be necessary for the formation of a flagellum (flagellar toolkit) were present in the preliminary draft genome of *T. philomaios* (Fig. 13).

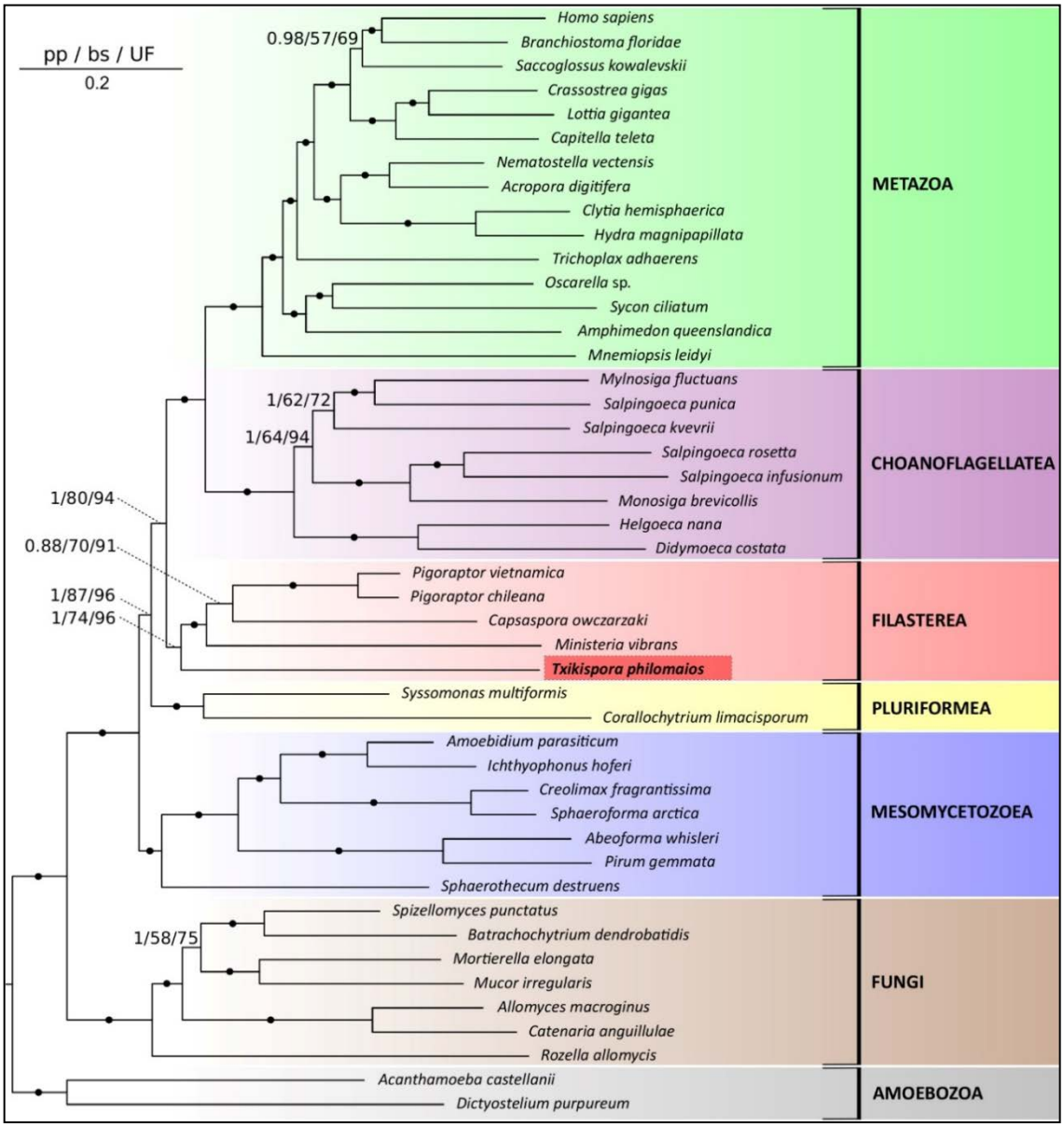


Fig. 12: Phylogenomic analysis using a concatenation of 96 conserved genes (29700 aminoacidic positions) places the novel amphipod parasite *T. philomaios* as the earliest evolving filasterean in clade Holozoa. The consensus phylogenetic tree was based on the branching obtained by Bayesian inference. The tree includes a selection of the main Opisthokont groups and unicellular holozoan lineages. Node support values are shown in clusters of three, representing Bayesian posterior probability (pp) run on Mrbayes, maximum likelihood bootstrap support (bs) generated using RAxML, and ML ultrafast bootstrap support (UF) from IQ-TREE respectively. Nodes with values (> 0.95 pp, > 95 bs, > 95 UF) are represented by a black dot on the branch.

The analysis indicated the presence of the genetic toolkit necessary to build a flagellum in both *Pigoraptor* species, which have been confirmed (SEM/TEM) to include flagellated cells among their lifecycle stages. In contrast, none of these genes occurs in the genome of non-flagellated *C. owczarzaki*. There is a single exception, the intra-flagellar transport A (IFT A) gene, which was not found in *P. vietnamica*'s draft genome. Additionally, the flagellated predator *S. multiformis* was observed to possess the complete flagellar toolkit as well, which is only partially complete in the other pluriformean species, *C. limacisporum*, which is a non-flagellated saprotroph.

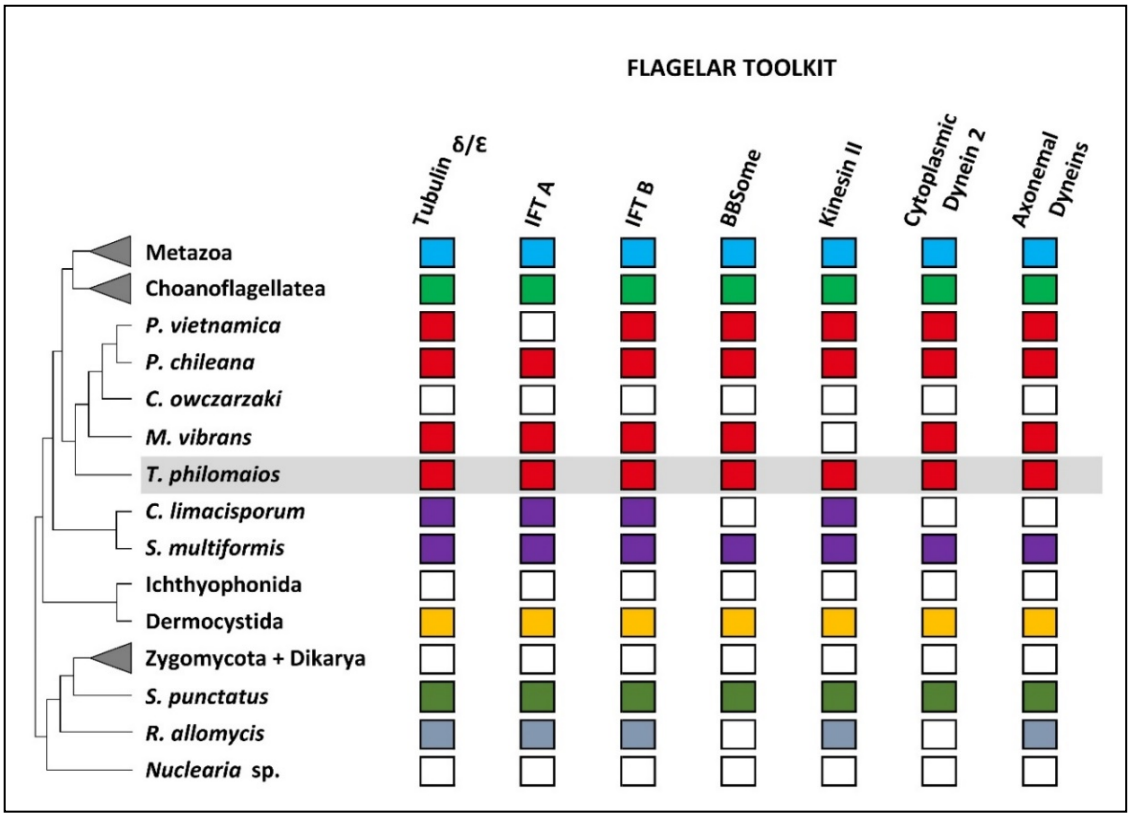


Fig. 13: Presence/absence of key genetic components of the flagellar apparatus in several lineages of Opisthokonta. Colour indicates presence of the gene specified above (tubulin δ/ε, intraflagellar transport A (IFT A), intraflagellar transport B (IFTB), Bardet–Biedl syndrome protein complex (BBSome), kinesin-II, cytoplasmic dynein–2, and axonemal dyneins); and a white space absence. Different lineages are indicated by distinct colours, and the phylogenetic tree to the left indicates relationship between those lineages. The figure has been adapted from Torruella et al. 2015 to include novel species *T. philomaios* (highlighted in grey), the two flagellated species in genus *Pigoraptor* sp. and the flagellated pluriformean *S. multiformis*. The tree has been modified to include *Pigoraptor* sp. and *S. multiformis* in the phylogenetic position established by Hehenberger et al. 2017, and *T. philomaios* according to our genomic analysis.

4. Discussion

4.1 Phylogeny and diversity

Until the recent addition of *Pigoraptor* by Hehenberger et al. (2017), Class Filasterea comprised only two genera: *Capsaspora* (*C. owczarzaki*) and *Ministeria* (*M. vibrans* + *M. marisola*). Hehenberger et al. (2017) also suggested the inclusion of an abundant group of marine opisthokonts "MAOP-1" (del Campo & Trillo 2013) into Filasterea. The ecology of this clade, formed by uncultured organisms, remains entirely undetermined except for an apparent inclination for the low-oxygen fraction of the water column in coastal waters of the Indian, Atlantic, and Pacific Oceans. Our results (Fig. 11) further support the inclusion of MAOP-1 in Filasterea. However, none of the ML analyses are conclusive, and the relative phylogenetic position of the group among existing filasterean species varies. In Hehenberger et al. (2017), MAOP-1 appeared as sister to *Pigoraptor* sp. (ML Bootstrap = 52%), but our analysis showed it as weakly sister to *Pigoraptor* spp. plus *C. owczarzaki* (in both cases with related environmental sequences) plus the LN****** environmental sequences. Our 18S phylogenetic analysis without environmental sequences (Fig. 10) also supported the inclusion of *T. philomaios* into Holozoa, but not its association with Filasterea.

The multigene-based phylogenomic analysis of *T. philomaios* indicates that the novel parasite is indeed a filasterean as shown by the environmental-enriched 18S phylogenetic tree in Fig. 11. In fact, the multigene tree supports the basal position of the novel parasite within the clade, supplanting *Ministeria* sp. as the earliest-diverging lineage in the Class. The tree, consistent with existing holozoan phylogenomic trees and retrieves with strong support the classes Choanozoa, Filozoa, and Pluriformea. However it does not settle the dispute around the phylogenetic position of Pluriformea as sister to Filozoa or to Icthyosporea (Torruella et al. 2015, Hehenberger et al. 2017); since part of the concatenation used in (Hehenberger et al. 2017) is used for constructing the tree in Fig. 12.

It is well established that single-gene trees are unable to resolve deep eukaryotic phylogenetic relationships. This is particularly evident for holozoan relationships, as shown by Simion et al. (2017) among others. Our results suggest that the use of uncharacterized environmental sequences in phylogenetic studies based on 18S provide additional phylogenetic information that may assist in resolving evolutionary relationships of novel holozoan organisms, as has previously been demonstrated for other eukaryotic groups and eukaryotes as a whole (e.g. Berney et al 2004; Cavalier-Smith 2004; Bass et al. 2018; Hartikainen et al. 2016).

Several environmental sequences were closely related to existing filasterean species (Fig. 11). The uncultured sequence Metagenome seq. OBEP011433235 most likely belongs to a novel *Pigoraptor* sp. species, which evidences the preference of the genus for the sediments of stagnant freshwater systems, and a global distribution (Denmark, Chile, and Vietnam). However, the environmental sequences FPLL01002905 and FPLS01019718, although sister to *C. owczarzaki*, are too distantly related to sensibly infer any lifestyle or other phenotypic similarity between them and *Capsaspora*. Interestingly, its occurrence in a Danish grassland (Karst et al. 2016), contrasts with the rest of environmental sequences associated to Filasterea, which were sampled from aquatic ecosystems. Although it is not possible to determine whether other filasterean environmental sequences are parasites, other symbionts, or free-living, our discovery of a true filasterean parasite means that this is now a realistic working hypothesis.

At some point in the evolutionary history of their lineages, *C. owczarzaki* and *T. philomaios* evolved endosymbiotic and parasitic behaviours closely associated with host haemolymph and haemocytes, highly uncommon target cells/tissues in the related clade Ichthyosporea (Glockling et al. 2013). Whether filasterean radiation preceded that of early metazoans 650-833 million years ago (Paps 2018) remains unresolved. Nonetheless, a common tissue trophism could suggest certain predisposition in the early ancestors of filastereans to colonize the haemolymph (or precursor cells) of other organisms, that could be shared by related uncultured filastereans. Actually, tissue specificity is often determined by evolutionary changes occurring early in a lineage. For instance, in ichthyosporeans a different tissue trophism allows to differentiate between the two orders (Mendoza et al. 2002), but it also happens in other protist clades in and out Holozoa; as it is the case of myxozoans (Molnár and Székely 2014) or apicomplexans (Leander et al. 2006).

4.2 Clinical signs and histopathology

T. philomaios cells congest the host's haemolymph and tegument, making heavily infected amphipods present a light-yellow colouration and reduced carapace transparency (internal organs are not easily visible though the carapace). Definite colour alterations of the host's carapace have been documented for other parasitic infections, such as those produced by acanthocephalans, cestodes, and trematodes (Lagrue et al. 2016; Johnson & Heard 2017). Other micro-eukaryotic cells targeting tegument and haemolymph in amphipods (*Haplosporidium* sp.) have also been associated with a pallid carapace and opacity. However, amphipods with heavy haplosporidiosis look whitish rather than yellowish, at least in *Echinogammarus* sp. and *Orchestia* sp. (Urrutia et al. 2019). The formation of cell aggregates, very evident in fresh

haemolymph smears, is characteristic among filastereans (Sebé-Pedrós et al. 2013) and facilitates the differentiation between *T. philomaios* and other protistan parasites with similar size. We have also observed infected hosts to be more sessile and unresponsive to stimuli, but this is the case for other protist parasite infections as well, not only in amphipods (Feist et al. 2009; Lefèvre et al. 2009).

Measuring less than 3 µm in diameter *T. philomaios* is one of the smallest known holozoans. In clade Filasterea only the bacterivorous *M. vibrans* would have a similar size, with its round cells being 2.1-3.6 µm in diameter (Mylnikov et al. 2019). The highly motile predators *P. vietnamica and P. chileana* tend to be considerably bigger (5-12 µm), in the size range of most choanoflagellates and corallochytreans (Raghu-kumar 1987; Dayeland King 2014; Tikhonenkov et al. 2020). Only the zoospores of few species of ichthyosporean parasites such as *Sphaerothecum destruens* or *Dermocystidium percae* have been reported to have a similar or even smaller size than *T. philomaios* (Pekkarinen & Lotman 2003, Andreou et al. 2011). A reduced body and genome size have been linked to parasitism in other protistan groups such as microsporidians or myxozoans (Keeling & Fast 2002; Keeling 2004; Holzer et al. 2018). This has not been studied for unicellular holozoans, possibly due to the absence of parasites among choanoflagellates, and rarity of free-living forms in Ichthyosporea (Mendoza et al. 2002; Glockling et al. 2013; Hassett et al. 2015). Filasterea now includes free-living, symbiotic, and parasitic species, making it a good candidate for such comparative analyses, especially when ecological and genomic data from the group's uncharacterised diversity are elucidated. The presence of holozoan protists with reduced genomes could provide very valuable information, as many studies focus on gene gains and losses to understand how and when animal multicellularity evolved (Paps et al. 2013; Grau-Bove et al. 2017; Richter et al. 2018).

4.3 Ultrastructure

Ultrastructurally, a crown of microvilli around a single flagellum makes choanoflagellates the most easily identifiable of all unicellular holozoans (Mah et al. 2014). The presence of a single posterior flagellum is a hallmark trait among opisthokonts (Cavalier-Smith 1987) and also been observed in holozoan Classes Filasterea, Corallochytrea and Ichthyosporea (Marshall et al. 2008; Torruella et al. 2015; Hehenberger et al. 2017; Mylnikov et al. 2019).

Fresh smears of *T. philomaios* showed the presence of cell-projections comparable to the flagellar structures described by light microscopy and TEM in *M. vibrans* and *Pigoraptor* sp. (Torruella et al. 2015; Hehenberger et al. 2017; Mylnikov et al. 2019). Additionally, the analysis of *T. philomaios´* genome shows the presence of all the genes deemed necessary to develop a

flagellum. Possession this "flagellar toolkit" does not necessarily mean expression of this structure, but there is certainly a demonstrated correlation between the presence of these genes and the occurrence of a functional flagellar structure (Torruella et al. 2015, Hehenberger et al. 2017). Besides, genes that are not under a selective pressure tend to quickly degenerate (Xing & Lee 2006). However, no definitive evidence of a flagellum was observed in the histopathological analysis, and we only have limited ultrastructural evidence of its occurrence by TEM (Fig. 7J). While inconclusive, we must note that in fresh smears *T. philomaios* cells were exposed to a substrate and marine water, but histology and TEM analysed them fixed in tissues and haemolymph. The zoospores of dermocystids are the only known flagellated stage among parasitic/endosymbiotic holozoan protists, and quickly lose the flagellum after penetrating into the host (Pekkarinen et al. 2003). Besides, the flagellum of *M. vibrans* was only observed after examination of over 1,000 cells (Mylnikov et al. 2019), a number not reached for *T. philomaios*, which has also resisted culturing attempts (see below). A non-flagellated *T. philomaios* would imply a secondary loss of the structure (based in our phylogeny, Fig. 11, 12), the second one within Filasterea after *C. owczarzaki*. Two losses are less parsimonious but could strengthen the idea of a parasitic/endosymbiotic lifestyle driving them, which has also been suggested for non-flagellated ichthyosporean parasites in order Ichthyophonida (Marshall & Berbee 2011, Torruella et al. 2015).

Microvilli are actin-based filopodial structures present in filozoans (Karpov et al. 2016; Sebé-Pedrós et al. 2017; Mylnikov et al. 2019). They form a crown around the flagellum in choanoflagellates and are evenly distributed around the cell in all filastereans and pluriformeans (Mylnikov et al. 2019; Tikhonenkov et al. 2020), clades in which they can be up to three or four times the length of the cell (10 µm in *M. vibrans*, 20 µm in *C. owczarzaki*, and 34 µm in *S. multiformis*). However, they are not present in cystic and dividing stages, what could explain the reduced evidence for them in *T. philomaios* (Fig. 7I). Moreover, their occurrence was not noticed in the original descriptions of *C. owczarzaki* done on explanted pericardial sacs of snails (Owczarzak et al. 1980), but they are evident when the facultative symbiont is in axenic culture (Sebé-Pedrós et al. 2013), where they have been shown to facilitate movement, cell-cell adhesion, and food particle capture (Parra-Acero et al. 2020). It is possible that microvilli are not desirable in the haemolymph of a host, where the current impedes movement and there is no substrate surface other than target haemolymph cells.

Opisthokonts are characterized by flat non-discoid cristae (Cavalier-Smith & Chao 1995), with ichthyosporean *Ichthyophonus hoferi* being one of the few exceptions (Spanggaard & Huss 1996). Mitochondria in *T. philomaios* follows the norm and possesses lamellar cristae (Fig. 7G,

7I). The radial distribution of numerous mitochondria in the periphery of non-cystic stages (Fig. 6A) could indicate a close in time cell division between daughter cells, as observed in the ichthyosporean *Sphaerothecum* sp. (Borteiro et al. 2018). In contrast, the absence of mitochondria in stages with a thicker wall suggests a resistant spore-like stage, as it is the case in the ichthyosporean *Amphibiocystidium* sp. (González-Hernández et al. 2010). However, the structure and activity of mitochondria in parasites has been observed to be extremely flexible (Zíková et al. 2016), as they would be able to use mitochondrial metabolites of the host (de Melo & Souza 1992).

Numerous electron-dense bodies comparable to those observed in other filasterean species (Owczarzak et al. 1980, Tikhonenkov et al. 2020) are scattered in the cytoplasm of *T. philomaios* (Fig. 6A, 6C, 6D, 7G, 7H, 7K). However, their occurrence is not characteristic of filastereans or even holozoan protists, as they have been observed in distantly related clades such as apicomplexans, ascetosporeans, or dinoflagellates (Speer et al. 1999; Stentiford & Shields 2005; Feist et al. 2009). Nevertheless, their size and number has been suggested to be of taxonomic value in Icthyosporea (Pereira et al. 2005), and indicative of the function of certain life stages and their maturation (Vilela & Mendoza 2012; Fagotti et al. 2020). These bodies have been described as lipid globules in *M. vibrans* (Mylnikov et al. 2019) and reserve substances (most likely glycoprotein) in genera *Pigoraptor* and *Syssomonas* (Tikhonenkov et al. 2020). In contrast, the occurrence of a double lipidic layer around them in *C. owczarzaki* made Owczarzak et al. (1980) suggest that these "lipid filled vacuoles" were excreted. In *T. philomaios* we observe two main forms; the first is a smaller and electron-lucent body similar to those observed in genera *Ministeria*, *Pigoraptor*, and *Syssomonas*. The second form is a larger and electron-dense body surrounded by a double lipid layer (Fig. 6H, 7G) that appears to be excreted (Fig. 7H) as proposed for *C. owczarzaki*. However, its implication in the formation of the cell wall should be considered, as it is not clear how the ejected material could trespass the outer membrane (Fig. 7G).

4.4 Life cycle and potential hosts

So far, all filastereans have been culturable (Stibbs et al. 1979; Cavalier-Smith and Chao 2003; Hehenberger et al. 2017; Mylnikov et al. 2019), allowing a detailed description of their life cycle in culture conditions. In contrast, *T. philomaios*, like most parasites in the clade Ichthyosporea remains unculturable (Cafaro 2005; Glockling et al. 2013). According to the diagnostic description of Class Filasterea Cavalier-Smith 2008, trophic stages in this lineage do not possess a cell wall (Shalchian-Tabrizi et al. 2008). In free-living genera *Pigoraptor* and *Ministeria*, this

non-walled stage corresponds to a flagellated amoeba which uses its retractile microvilli to capture preys and attract food particles (Hehenberger et al. 2017; Mylnikov et al. 2019). In turn, trophocytes of the endosymbiont *C. owczarzaki* lack a flagellum, and even microvilli if cultured in explanted tissues of *B. glabrata* (Owczarzak et al. 1980; Sebé-Pedrós et al. 2013). Although morphologically different, the behaviour of trophocytes is the same in all known filasterean species; they can either divide by binary fission or encyst when the food source is depleted (Hertel et al. 2002; Tikhonenkov et al. 2020). The binary fission observed by light microscopy in few walled cells of *P. vietnamica* (Tikhonenkov et al. 2020) represents the only known exception of cellular division occurring outside the trophic stage. Interestingly, our TEM analysis indicates that quite the contrary occurs for *T. philomaios*, in which cell division appears to occur exclusively inside walled cells (Fig. 6B, 6I, 7D) as in Corallochytrea and Ichthyosporea (Raghu-Kumar 1987; Lotman et al. 2000; Pekkarinen et al. 2003; Glockling et al. 2013). If flagella and/or microvilli occur in *T. philomaios* trophocytes (Fig. 7I, 7J), these structures are likely lost when parasitic cells either penetrate or are engulfed by host haemocytes (Fig. 7K).

A single host haemocyte can contain up to ten *T. philomaios* cells, in which four walled endospores arise inside walled parent cells (Fig. 7D). Comparable cellular structures containing 16-32 endospores are the result of a palintomic division in corallochytrean cystic stages (Raghu-Kumar et al. 1987; Tikhonenkov et al. 2020). Once mature, *T. philomaios* endospores would leave the parent cells through an opening formed in its wall, by which time its thickness is much reduced, as in Corallochytrea and Ichthyosporea (Mendoza et al. 2002; Marshall & Berbee 2011; Tikhonenkov et al. 2020). The wall thickness, electron-density and amount of reserve material vary greatly among endospores. Some cells appear active even before exiting the ruptured parent cell (Fig. 7E), presumably ready to re-infect other haemocytes and tissues in the same host, as it has been shown for several ichthyosporeans (Arkush et al. 2003; Marshall et al. 2008; Kocan 2019). Other cysts seem to be resistant (Fig. 6D, 6F), perhaps capable of infecting other amphipods or even remaining viable in the environment for months (Marshall & Berbee 2010; Gozlan et al. 2014; LaPatra & Kocan 2016).

The transmission method for *T. philomaios* cells is unknown, as for *C. owczarzaki* (Harcet et al. 2016), and most parasites in Ichthyosporea (Glockling et al. 2013). A direct cycle by consumption of infected prey has been demonstrated in the ichthyophonid *I. hofferi* (Kramer-Schadt et al. 2010), and could be possible for *T. philomaios*, given the high levels of inter-specific predation (Dick et al. 1999), cannibalism (Kinzler & Maier 2003), and scavenging of conspecifics (Agnew & Moore 1986) observed in amphipods. The thicker ameboid endospores observed in *T. philomaios* are also remindful of the infective waterborne cells observed in ichthyophonid

parasites (Olson et al. 1991, Andreou et al. 2009, Kocan 2019), which unlike those in order Dermocystida, lack a flagellum (Mendoza et al. 2002). Additionally, cysts of the so called "TMS" ichthyosporean infecting *Tenebrio molitor*, persist in the connective tissues associated to the gonads, and are transmitted with sperm during copulation (Lord et al. 2012). The presence of few *T. philomaios* cells infecting amphipod gonads throughout the year (although with low prevalence = 1.9%) leaves open the possibility of a similar "nuptial transmission" for the novel parasite. In that case, *Echinogammarus* sp. and *Orchestia* sp. would represent the reservoir for *T. philomaios*, which according to the most extended definition is an environment/population where the pathogen can be permanently maintained and transmitted (Haydon et al. 2002).

Finally, an indirect transmission cycle has been contemplated as well, given the generalist infectivity observed in *T. philomaios* and ichthyosporean parasites (Andreou et al. 2012; Rowley et al. 2013; Combe and Gozlan 2018). Copepods have been proposed as the missing intermediate host for the fish parasite *I. hofferi*, which infects herring and salmon species (Hershberger et al. 2002; Gregg et al. 2012). Interestingly, harpacticoid copepods are some of the most common invertebrates co-occurring with amphipods in the upper part of the intertidal in Newton's Cove, Camel, Dart and Tamar estuaries (personal observation; Hicks & Coull 1983). However, our PCR based search for *T. philomaios* in copepods (n = 1300 individuals) was negative, just like the histopathological analysis. In turn, the results for the turbellarian *Procerodes* sp. were PCR positive during May. The platyhelminth, which is very common in the North Atlantic, appears to predate on diseased *Echinogammarus* sp. prey and carcasses (Den Hartog 1968; Taylor 1986), showing a link and a possible role as intermediate host. A more extensive histopathological analysis of *Procerodes* sp. will be necessary to substantiate its possible role as intermediate host of *T. philomaios*. If uninfected the turbellarian could still be a vector helping the dispersal of viable *T. philomaios* cysts.

4.5 Distribution, prevalence, and ecological significance

The low number of filasterean species and their rare appearance in environmental samplings has prevented any previous estimation of their temporal prevalence, as it has been assayed for larger holozoan clades Ichthyosporea and Choanoflagellatea (Marchant & Perrin 1990; Kasesalu et al. 2000; Pekkarinen & Lotman 2003). The prevalence of *C. owczarzaki* in *Biomphalaria glabrata* has been observed to vary from 1% to 45% depending on the strain (Hertel et al. 2002), but the measurement, done on cultured snails, does not estimate occurrence on a time period. Our study is the first one to reveal a temporal pattern in the abundance of a filasterean species. The quickly vanishing peak in prevalence observed for *T. philomaios* during May, exposes

seasonality as an until now unaccounted bias for the scarcity of filasterean sequences in environmental samplings (del Campo et al. 2015; Hehenberger et al. 2017; Mylnikov et al. 2019). A similar short temporary window in the transmission of *C. owczarzaki* between snails, could explain, at least partially how it has eluded sampling efforts to find it in the wild (Ferrer-Bonet & Ruiz-Trillo 2017). Additionally, our failed efforts to amplify the 18S of *T. philomaios* from filtered water collected in Newton's Cove during May, reinforces the hypothesis of a reduced detection capability of eDNA for parasites/endosymbionts (Dumonteil et al. 2018).

So far, it has been observed that *T. philomaios* is able to infect at least two different amphipod genera, indicating certain range of hosts specificity that could expand notably if infection in the turbellarian *Procerodes* sp. is substantiated by histology. In this study, the prevalence of *T. philomaios* was as high as 64% (May 2016), with about a third of the infected individuals presenting heavy infections associated to tissue disruption and haemolymph congestion by parasitic cells. From the point of view of pathology, other protistan parasites that tend to multiply and congest the haemolymph of crustacean hosts, such as the dinoflagellate *Hematodinium* sp. have been associated with a reduced oxygenation capability and diminished overall fitness (Taylor et al. 1996; Stentiford et al. 2001). The observed unresponsiveness to stimuli in infected amphipods is consistent with the systemic damage observed in the integument, which functions as the sensorial system (Steele & Oshel 1987). Collectively, numerous protistan parasites have been found to profoundly alter the populations of amphipods and other crustaceans (Morado 2011; Ironside & Alexander 2015). Considering that several ichthyosporean parasites are responsible for important mortality events in fish and amphibian populations (Raffel et al. 2008; Kirkbright et al. 2016) it would be interesting to monitor the influence of *T. philomaios* in the amphipod population, as *Echinogammarus* and *Orchestia* are amongst the most common and abundant crustaceans in coastal ecosystems of Northern Europe (Marques & Nogueira 1991; Mantzouki et al. 2012), and important invasive species outside the continent (Van Overdijk et al. 2003; Herkül et al. 2006).

5 TAXONOMIC SUMMARY

Eukaryota Chatton, 1925 / Eukarya Margulis & Chapman, 2009: Opisthokonta Adl, 2005: Holozoa Adl, 2012: Filasterea Shalchian-Tabrizi, 2008: Ministerida Cavalier-Smith, 1997

5.1 Family Txikisporidae Urrutia, Feist & Bass n. fam.

***Diagnosis*.** Naked unicellular and uninucleated protists morphologically similar to individuals in family Ministeriidae Cavalier-Smith 2008, but with a parasitic lifestyle.

Type genus. Txikispora n. g. (see below)

Genus *Txikispora* Urrutia, Feist & Bass n. g.

Etymology. 'txiki': small and 'spora': a seed (Basque). The name has been chosen to reflect relatedness with the filasterean endosymbiont *Capsaspora* Hertel, 2002 ("the quick eating seed") and its small size, while putting a distance with other small spore forming parasitic lineages with Latin stems.

Diagnosis*.* As for species (see below)

Type species. *Txikispora philomaios* (see below)

Txikispora philomaios Urrutia, Feist & Bass n. sp.

Etymology*.* Txiki-: small, spora: spore, philo-: lover, maios: the month of May. "The little May-loving spore", referring to its predominant detection (as a parasite of amphipods) in that month.

Diagnosis. Virtually spherical monokaryotic stages, with a length of 2.6 ± 0.41 µm and a width of 2.26 ± 0.34 µm. The round and walled multinucleated stage contains four walled cells inside, which resemble a lot the monokaryotic stages. The size of this divisional stage is slightly bigger (3.17 µm ± 0.24 in diameter). Infection develops principally inside host haemocytes and connective tissues, especially those associated to the integument. Infection in amphipods in the southwest of England occurs consistently during late April and May, the prevalence of the parasite during the rest of the year is anecdotic (<2%). The parasite has been also linked to the gonads, being the only organ that appears to be infected during the rest of the year. There is host reaction to the parasite in form of melanization and granuloma formation, especially when the parasite affects the hepatopancreas.

Type host*.* Amphipods *Echinogammarus* sp. and *Orchestia* sp**.**

Type location. Coastal waters in Newton's Cove (UK)

Type material. Original slides used for this paper are stored together with biological material embedded in wax and epoxy resin in Cefas Weymouth Lab. Type material is stored as RA16020 (specimen no. 19) and RA17028 (specimen no. 53) and (specimen no. 287). The SSU rDNA sequence is deposited in GenBank under accession number (to be submitted).

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CHAPTER 3

Ultrastructure, Phylogeny and Histopathology of Two Novel Haplosporidians Parasitising Amphipods, and Importance of Crustaceans as Hosts

Abstract

This study provides morphological, ultrastructural and phylogenetic characterization of two novel species of Haplosporidia (*Haplosporidium echinogammari* n. sp. and *Haplosporidium orchestiae* n. sp.) infecting amphipods of the genera *Echinogammarus* and *Orchestia* collected in the south west of England. Both parasites infect the connective tissues associated with the digestive gland and the tegument, and eventually infect other organs causing disruption of host tissues with associated motor impairment and fitness reduction. Prevalence of infection varied with host species, provenance, and season, being as high as 75 % for individuals of *E. marinus* infected with *H. echinogammari* in June (n = 50). Although no spores were found in any of the infected amphipods examined (n = 82), the morphology of monokaryotic and dikaryotic unicellular stages of the parasites enabled differentiation between the two new species. Phylogenetic analysis of the new species based on the SSU rDNA gene placed *H. echinogammari* close to *H. diporeiae* in haplosporidian lineage C, and *H. orchestiae* in a novel branch within *Haplosporidium*. Genetic diversity of the haplosporidians infecting these and other amphipod species was evaluated and compared to morphological and ultrastructural changes to host tissues. The phylogenetic relationship of haplosporidian infections in other crustacean hosts is discussed after inclusion into the analysis of 25 novel SSU rDNA sequences obtained from crabs, isopods and crayfish.

KEY WORDS: *Echinogammarus*, *Orchestia*, Crustacea, *Haplosporidium echinogammari, Haplosporidium orchestiae*, host parasite association, life cycle, phylogeny

1. Introduction

The order Haplosporida (Caullery & Mesnil 1899), within the class Ascetosporea (phylum Endomyxa), comprises four genera (*Minchinia, Bonamia, Urosporidium* and *Haplosporidium*), all small endoparasites of marine and freshwater invertebrates (Hartikainen et al. 2014). Several species within the clade are well known parasites of bivalves, causing recurrent mortality events (Haskin & Andrews 1988) that decimate natural and farmed populations (Ford & Figueras Huerta 1988). Notorious examples of substantial economic losses associated with haplosporidian infections include the decline of eastern oyster (*Crassostrea virginica*) populations in the East Coast of North America due to *Haplosporidium nelsoni* (MSX disease) and *Haplosporidium costale* (Ford & Tripp 1996). Infection with *Bonamia ostreae* was a major factor associated with the collapse of *Ostrea edulis* production in Europe in the last decades of the twentieth century (Pichot et al. 1979, Friedmann & Perkins 1994). In contrast to economic losses affecting aquaculture, the ecological significance of haplosporidian infections is more difficult to determine. However, there is significant evidence of the impact of haplosporidians on non-cultured species from a wide range of environments. Infection with *Haplosporidium pinnae* appears to be a key factor in the decline of the fan mussel (*Pinna nobilis*) in the Mediterranean Sea (Trigos et al. 2014, Catanese et al. 2018). In addition, there may be reduced bioturbation of sediments as a consequence of polychaetes infected by *Haplosporidium parisi* and *Haplosporidium scolopli* (Paramor & Hughes 2004, Ormières 1980), or even changes in the population structure of important invertebrate predators such as the common shore crab *Carcinus maenas* infected by *Haplosporidium littoralis* (Stentiford et al. 2013).

The taxonomic relationships within Haplosporida remain a challenge more than a century after its discovery. Currently, there are approximately 54 described haplosporidian species with approximately 20 unnamed organisms and at least another 50 uncharacterized sequences distributed within or related to one of the four genera constituting the order Haplosporida. Despite advances in the understanding of the phylogeny of the group following the introduction of electron microscopy in the 1950s and molecular techniques in the last 25 years (Cavalier-Smith 1993, Berthe et al. 2000, Hartikainen et al. 2014), aspects of the life-cycle, diversity, ecology, and even morphological features remain poorly understood. Haplosporida are parasitic protists having multinucleate plasmodia and ovoid-walled spores lacking a polar filament and with an orifice at one pole (Perkins 2000). Historically, divisions between genera have been based on spore ornamentation. *Urosporidium* is clearly distinguished from the others by having an internal flap of wall material covering the orifice of the spore. Morphological

differentiation between *Minchinia* and *Haplosporidium*, both with an external hinged lid, proved more difficult due to failure to find new comparative type material of both genera, which were described in the early years of the twentieth century. In addition, the presence of apparently non-spore-forming *Bonamia* in the order muddied the description until the first spore-forming *Bonamia* sp. was found (Carnegie et al. 2006). Ormières (1980) proposed separating *Minchinia* and *Haplosporidium*, based on the origin of the spore ornamentation. Spore ornamentation composed of epispore cytoplasm would define genus *Minchinia*, while spore-wall formed ornamentation would define genus *Haplosporidium*. Molecular analyses (Reece et al. 2004) support this ontogenic hypothesis and confirm the monophyly of *Urosporidium*, *Bonamia*, and *Minchinia* genera. However, *Haplosporidium* is currently paraphyletic (Burreson & Reece 2004, Hartikainen et al. 2014, Ward et al. 2018). These authors highlight the need for taxonomic revisions based on molecular characterisations, histopathological and ultrastructural descriptions to facilitate the erection of novel monophyletic genera. Attention is increasingly being focused on haplosporidian infections in non-molluscan invertebrates, including crabs and amphipods as definitive or intermediate hosts.

Amphipoda is a major order of ubiquitous malacostracan crustaceans, characterized by the lack of carapace, differentiated limbs or “poda” and a medio-lateral flattening of the body. The adaptability, resilience to abiotic fluctuations, and a wide spectrum of feeding strategies developed by these benthic crustaceans, have allowed them to colonize some of the most demanding and hostile environments, including polar regions (Poltermann 2001) and hydrothermal vents (Sheader et al. 2004). With almost 10,000 species, mainly in marine ecosystems, the role played by scavenging and detritivorous amphipods as secondary producers (Norderhaug & Christie 2011), decomposing and recycling organic matter back to the food web (Wilson & Wolkovich 2011), makes them a dominant component of many benthic macroinvertebrate assemblages (MacNeil et al. 1997). Predictably, being extremely abundant in a wide range of ecosystems makes amphipods an important part of the diet for other crustaceans, fish, birds, and even mammals (Garrison & Link 2000, Bocher et al. 2001, Holst et al. 2001). They are also used as a protein source in aquaculture (Moren et al. 2007). In addition to their diversity, abundance, and ecological importance, amphipods are widely used in biomonitoring (Fialkowski et al. 2003) and toxicological studies (Hanna et al. 2013). Yet, there is a notable lack of information and understanding of amphipods as vectors, reservoirs and primary hosts for a number of parasites.

Although molluscs are the best-known hosts of haplosporidian parasites, awareness of the role played by crustacean hosts has increased in recent decades (Hine et al. 2009). Some of

these relationships potentially have ecological and commercial importance, as haplosporidians infect several species of crabs (Stentiford et al. 2013) and shrimps (Bower & Meyer 2002, Utari et al. 2012). However, there is very limited information on Haplosporida infecting amphipods. The first of these to be described, *Haplosporidium gammari* (later reclassified as *Claustrosporidium gammari* due to the lack of pore in the spore (Larsson 1987)), infected *Rivulogammarus pulex* sampled near Louvain in Belgium (Van Ryckeghem 1930). It took fifty years before another infected *R. pulex* was studied (Larsson 1987) and there are no gene sequences for this parasite. More recent discoveries include *Haplosporidium diporeiae* infecting the benthic amphipod *Diporeia* sp. in the Laurentian Great Lakes in the USA (Winter & Faisal 2014) and haplosporidian-like parasites infecting *Parhyale hawaiensis* collected from Sharm El-Nagha, in the Egyptian coast of the Red Sea (Ismail 2011). Ecological implications of haplosporidians infecting amphipods have been proposed in a recent study (Cave & Strychar 2014), suggesting a potential association between *H. diporeiae* infection and amphipod population declines in the Great Lakes since the late 1980s (Nalepa et al. 2007).

In this study, we describe the infection of amphipods by two novel species of *Haplosporidium*. The first species was found infecting amphipods of the genus *Orchestia* collected from Tamar and Dart estuaries (southwest of the United Kingdom), and Butrón estuary, in northern Spain. The second species was detected in *Echinogammarus marinus* sampled from Newton's Cove (Weymouth, UK). We provide histological, ultrastructural and phylogenetic information for both new species.

2. Materials and methods

2.1 Sample collection

Amphipods belonging to the genera *Orchestia* and *Echinogammarus* were collected from the Dart Estuary (Dittisham, Devon, UK), Newton's Cove (Weymouth, Dorset, UK), and the Butrón estuary (Plentzia, Spain) in 2016 and 2017, as shown in Table 1.

2.2 Histology and transmission electron microscopy

Amphipods were kept alive and dissected within 3-4 hours post collection. The head and anterior part of the thorax were immediately fixed in 100 % molecular grade ethanol. The proximate segments of the thorax of about 2 mm in size, were placed in 2.5 % Glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for electron microscopy. The remainder of the body, which included the last 3-4 segments of the pereon and the pleon, were fixed in Davidson's seawater fixative (Hopwood 1996) for 24 hours, then transferred to 70 % ethanol.

Table 1. Sampling information for the amphipod species collected by this study. Specific coordinates are provided for the exact sampling site together with the locality and the habitat (environment).

No	Sampling site	Locality	Environment	Niche	Date	Taxon	n	Coordinates
1	Newton's Cove	Weymouth, UK	Marine water	Upper shore, underneath rocks	April-16 to Dec-17	*E. marinus*	30 (every month)	50° 36' 17'' N, 02° 27' 03'' W
2	Dart estuary	Dittisham, UK	Brackish water	Upper shore, underneath rocks	27-Apr-17	*Orchestia* sp.	30	50° 23' 21'' N, 03° 35' 36'' W
3	Dart estuary	Dittisham, UK	Brackish water	Upper shore, underneath rocks	27-Apr-17	*E. marinus*	30	50° 23' 21'' N, 03° 35' 36'' W
4	Butrón estuary	Plentzia, Spain	Brackish water	Upper shore, underneath rocks	30-Aug-17	*Orchestia* sp.	30	43° 24' 25'' N, 02° 57' 23'' W
5	Oder river	Gryfino, Poland	Fresh water	Water column/sediment on the embankment	23-Jun-15	*Pontogammarus robustoides*	122	53° 15' 09'' N, 14° 26' 53'' E
6	Bug river	Poręba-Kocęby, Poland	Fresh water	Water column/sediment on the embankment	21-Jun-15	*Gammarus varsoviensis*	109	52° 41' 28'' N, 21° 41' 12'' E

For histology, Davidson's fixed samples were processed from ethanol to wax in a vacuum infiltration processor using established laboratory protocols (Stentiford et al. 2013a). Tissue sections were cut at a thickness of 2.5-3 µm on a Finnese® microtome, left to dry for 24 hours after mounting on VWR™ microscope slides and stained with H&E (Bancroft & Cook 1994). Cover-slipped sections were examined for general histopathology by light microscopy (Nikon Eclipse E800). Digital images and measurements were obtained using the Lucia™ Screen Measurement software system (Nikon, UK). Measurements of unicellular parasite stages were performed only for those showing good fixation and clear structure and for plasmodia which were not compressed by adjacent host tissues. Statistical analysis for normality and comparison between measurements was conducted in RStudio™. The level of infection was assessed using a scale ranging from 1 to 4, (1 - few parasite cells infecting few host tissues; 2 - unicellular and plasmodial stages in haemolymph and tegument; 3 - several organs and connective around them affected; and 4 - systemic infection often associated with important tissue disruption).

Four amphipod samples showing haplosporidian infections by light microscopy were selected for transmission electron microscopy (TEM) analysis. Glutaraldehyde-fixed samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 hour in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in epoxy resin 812 (Agar Scientific pre-Mix Kit 812, Agar scientific, UK) and polymerised overnight at 60 °C. Semi-thin (1 µm) sections were stained with 1 % Toluidine Blue and analysed by light microscope to identify target areas containing sufficient parasites. Ultrathin sections (70-90 nm) were framed on uncoated copper grids and stained with uranyl acetate and Reynold's lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using a GATAN Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.3 DNA extraction, polymerase chain reaction and sequencing

Amphipod gonad, gill, muscle, connective tegumental, nervous and digestive tissues were preserved in 100 % molecular grade ethanol (Fisher BioReagents™). Samples found to be infected via histology were selected for DNA extraction, PCR amplification, and sequencing. Infected tissues were disrupted and digested overnight using Fast Prep® Lysing Matrix tubes containing 0.2 mg (6 U) Proteinase K (Sigma-Aldrich®) diluted 1/40 in Lifton's Buffer (100 mM EDTA, 25 mM Tris-HCl, 1 % (v/v) SDS, pH 7.6). The following day, 1/10 (v/v) of 5 M potassium acetate was added, and tubes incubated on ice for 1 hour. From here DNA was extracted using the phenol-chloroform method described in (Chomczynski & Sacchi 1987). Partial SSU rDNA gene sequences belonging to the different haplosporidians were amplified by PCR as follows: A total reaction volume of 50 µl included (30.75 µl molecular water, 10 µL GoTaq® Flexi Buffer, 2.0 mM $MgCl_2$, 0.2 mm of each deoxyribonucleotide, 40 pM of each primer, 1.25 U GoTaq® Polymerase (Promega) and 200 ng of DNA. Cycling parameters: 3 min denaturation at 95 °C, followed by 35 cycles of 95 °C (30 sec), annealing (1 min) at 65 °C followed by (1 min) at 72 °C, amplicons extended by final incubation at 72 °C (10 mins), stored at 4 °C. Primers (Table 2), conditions and concentrations used for nested PCR followed Hartikainen et al. (2014). The resulting band (650 bp) was dissected and cleaned using 20 % polyethylene glycol 8000 (Sigma-Aldrich®) solution. A total volume of 15 µl of purified DNA with a concentration of (5 ng μl^{-1}) was mixed with 2 µl (10 µM) of forward primer (V5fHapl 5'- 3') and single-read Sanger sequenced (Eurofins®Genomics).

Table 2. Primers used for Haplosporidian PCR amplification, as in Hartikainen et al. (2014). Sequencing direction for forward primers (C5fHapl, V5fHapl) is (5'-3'), and (3'-5') for reverse primers (Sb1n, Sb2nHap).

Primer	Sequence	Specificity
C5fHapl	5' - GTAGTCCCARCYATAAACBATGTC - 3´	Haplosporidian SSU
Sb1n	3´ - GATCCHTCYGCAGGTTCACCTACG - 5´	Universal eukaryote
V5fHapl	5´ - GGACTCRGGGGGAAGTATGCT - 3´	Haplosporidian SSU
Sb2nHap	3´ - CCTTGTTACGACTTBTYCTTCCTC - 5´	Eukaryote (Haplo biased)

2.4 Sequence alignment and phylogenetic analysis

Haplosporidian sequences were confirmed by BlastN searches (Zhang et al. 2000) against Genbank nt database and by constructing preliminary trees. Sanger sequence chromatograms were edited by eye, and potentially aberrant nucleotides in highly conserved regions were replaced by an "N" when their quality scores were lower than Q30. Reference sequences from a comprehensive haplosporidian dataset (Hartikainen et al. 2014) were downloaded and aligned

with our own sequences in MAFFT (Katoh et al. 2017) using the L-ins-i algorithm, and the following parameters: 200 PAM/K=2 scoring matrix, 1.53 GAP opening penalty, with N having no effect on the alignment score. Sequences belonging to haplosporidian parasites deposited on GenBank after 2014 were also added. The alignment was edited in MEGA v7.0 (Kumar et al. 2016) and ragged ends cropped. Final refinement of the alignment was manually curated in Aliview (Larsson 2014). A maximum likelihood phylogenetic tree was constructed using RAxML Blackbox (GTR model with CAT approximation (all parameters estimated from the data); averages of 402 bootstrap values (MRE-based bootstopping criterion) were mapped onto the tree with the highest likelihood value (evaluated under GAMMA)) (Kozlov et al. 2019) on the Cipres Science Gateway (Miller et al. 2010). A Bayesian inference consensus tree was built using MrBayes v.3.2 (Ronquist et al. 2012) on Cipres using default likelihood model parameters except for the following changes: The number of substitution types was mixed; model for among-site rate variation, Invgamma; use of covarion like model, activated), MCMC parameters: 5 million generations and all compatible groups consensus tree. A final consensus tree was created on FigTree v1.4 (Rambaut 2014) based on the Bayesian topology. Following the same procedure, a second tree with different taxon sampling was generated including an additional 25 newly generated parasite sequences associated with crustacean hosts obtained from archive material from other projects, using the same PCR and analytical protocols as described above. No histological data were available for these 25 sequences.

3. Results

3.1 Clinical signs and prevalence

Haplosporidian infections in heavily infected *E. marinus* and *Orchestia* sp. were suggested macroscopically by whitish and opaque colouration of the body. Infection was also often associated with reduced jumping ability in the genus *Orchestia* and lethargic behaviour in *E. marinus*. When dissected, the haemolymph of severely infected individuals was more viscous and cloudier than that of uninfected individuals. Haplosporidiosis in *E. marinus* showed a distinct peak in prevalence during June/July, whilst prevalence of haplosporidiosis in *Orchestia* sp. was high in April (Fig 1.). For haplosporidian parasites infecting *E. marinus* in other locations and other amphipod genera, prevalence varied with the location and time of the sampling (Fig. 1). Only 1 out of 107 (0.93 %) individuals of *Gammarus varsoviensis* sampled from Western Bug in Poręba-Koceby showed a level of infection, while the prevalence was 3.2 % for the haplosporidian infecting *Pontogammarus robustoides* (n = 122) in Oder river as it passes near the town of Gryfino.

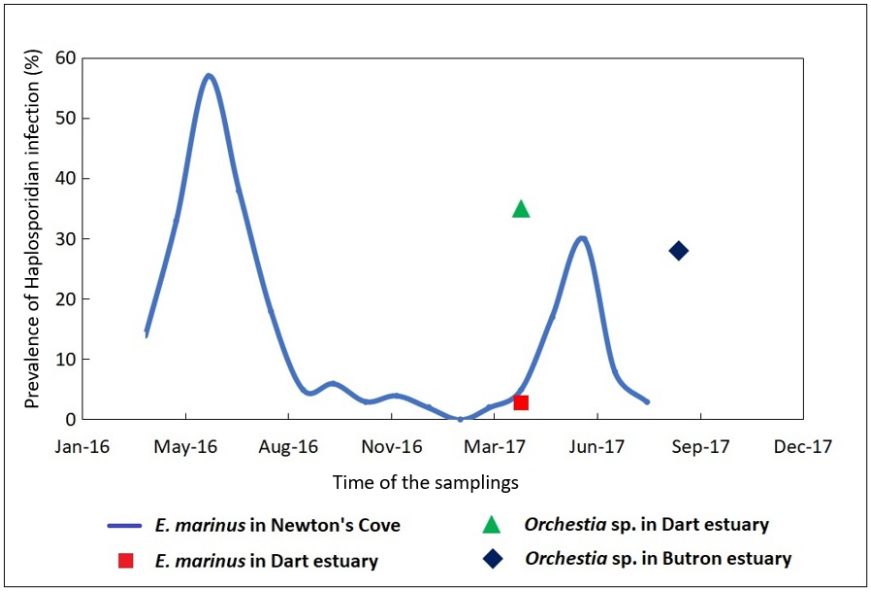

Fig. 1. Prevalence of haplosporidian infection (Y axis) for different times of the year (X axis) and amphipod species. Blue line shows prevalence in *E. marinus* sampled in Newton's Cove from April 2016 to August 2017. Prevalence for *E. marinus* sampled in Dart estuary (Dittisham) 27th April 2017 (red square). Prevalence for *Orchestia* sp. sampled in Dart estuary the 27th April 2017 (green triangle). Prevalence for *Orchestia* sp. sampled in Butrón estuary the 30th August 2017 (blue diamond).

3.2 Histopathology and ultrastructure

Light microscopy revealed morphological differences between infections in different hosts and locations. No spore stages were found in any of the infected amphipods examined (n = 82). Significant differences in length and width of the unicellular stages observed suggested two clearly differentiated taxa (Table 3). Transmission electron microscopy supported light microscopy observations, showing clear ultrastructural differences between the parasites *in E. marinus and Orchestia* sp. (Fig. 2 and Fig. 3).

In amphipod hosts with early haplosporidian infections, parasites were mainly located in the connective tissue, especially around intestine and hepatopancreas (Figs. 2A, 3A). The tegument was only lightly infected. During this phase of the infection, only plasmodial stages of the parasite were observed, most of them with fewer than 10 nuclei. As the intensity of infection increased, parasite stages were observed in the haemolymph (Figs. 2B, 3B), facilitating spread of infection throughout the body, with exception of the brain and peripheral nervous system.. In more intense infections, muscle and hepatopancreas were also affected and occasionally, extended disruption of the intestine and tegument was present (Fig. 2C). In general, unicellular monokaryotic and dikaryotic stages of the parasite were found mainly in the haemolymph and to a lesser extent in the tegument, while plasmodial stages tended to congest the connective

tissues around organs (Table 3). Chronic host response in the form of granuloma formation and melanization (Fig. 2C) in heavily infected individuals was observed. Haemocyte aggregations were noted for several infections regardless of the phase of the infection. In 15/17 selected amphipods (Table 3) there were sufficient (≥ 30) numbers of uncompressed plasmodia suitable for measurement. Sizes ranged from 6.6 ± 0.67 µm in parasite MK913655 infecting *E. marinus* in Newton's Cove in September 2016, to 9.4 ± 1.14 µm in parasite MK913668 infecting *Orchestia* sp. in Dart estuary during April 2017.

Morphometric analysis of monokaryotic and dikaryotic stages shows two well defined groups within parasites infecting both host groups (Fig. 4). The length of the monokaryotic parasite cells (n = 30) infecting *E. marinus* ranged between 2.49 ± 0.29 µm and 2.79 ± 0.39 µm, and width between 1.64 µm and 1.78 ± 0.19 µm. Monokaryotic stages of the parasites infecting *Orchestia* sp. were consistently larger with a minimum length of 3.46 µm and a maximum of 3.62 µm (st.dev. between 0.24 and 0.44 µm) and width between 2.04 and 2.32 µm (st.dev. between 0.15 and 0.36 µm) (n = 30). There are clear differences in the size and shape of dikaryotic stages of the two parasites. The elongated tube-like shape of cells in *E. marinus*, with a length ranging between 4.18 ± 0.31 µm and 4.72 ± 0.24 µm and a maximum width of 1.36 ± 0.12 µm (n = 30) contrast with the sub-spherical nature of the parasitic cells infecting *Orchestia* sp. which have a minimum width of 2.99 ± 0.28 µm and a length ranging between 3.51 and 3.97 µm (n = 30).

Ultrastructural differences between both groups (Fig. 2D, 2E, 2F and Fig. 3C, 3D) were evident in both unicellular and plasmodial stages. Plasmodial stages of the haplosporidian infecting *E. marinus* were more uniform, with slightly ovoid nuclei and small very condensed peripheral nucleolus, and several mitochondria (Fig. 2D, 2F).

Table 3. Individual amphipod samples showing haplosporidian infection. The reference number links to the phylogenetic tree in (Fig. 5), the sampling location, time of collection and host species. Information on the level of infection, tissues infected and parasite stage specificity for tissues can be found in the subdivision "Histopathology" of the table. Morphometric measurements of the plasmodial and unicellular stages are shown under "Parasite morphology".

GENBANK REFERENCE	HOST			HISTOPATHOLOGY				PARASITE MORPHOLOGY		
	Host species	Sampling location	Date	Level of infection	Tissues infected	Unicellular stages	Plasmodial stages	Plasmodium size (µm)	Unicellular stages size (µm)	Binucleated stages (µm)
MK913658	*E. marinus*	Newton's Cove (UK)	18-May-17	**4**	Tegument, connective and haemolymph	Haemolymph	Connective	7.14 ± 0.85	LENGTH: 2.56 ± 0.39 WIDTH: 1.79 ± 0.17	LENGTH: 4.25 ± 0.46 WIDTH: 1.38 ± 0.17
MK913659	*E. marinus*	Newton's Cove (UK)	18-May-17	**4**	Tegument, connective and haemolymph	Haemolymph	Connective	7.35 ± 1.00	LENGTH: 2.48 ± 0.29 WIDTH: 1.77 ± 0.24	LENGTH: 4.37 ± 0.48 WIDTH: 1.34 ± 0.15
MK913660	*E. marinus*	Newton's Cove (UK)	18-May-17	**3**	Mainly connective, especially around intestine and hepatopancreas. Tegument.	Absent	Connective and tegument	7.10 ± 0.76	–	–
MK913661	*E. marinus*	Newton's Cove (UK)	18-May-17	**1**	Connective tissue around intestine. Few plasmodia present in tegument	Absent	Connective and tegument	7.52 ± 1.20	–	–
MK913670	*P. robustoides*	Oder river (Gryfino, Poland)	23-Jun-15	**3**	Tegument and connective	Connective and Tegument	Connective and tegument	7.11 ± 0.90	Not measurable	Not measurable
MK913664	*P. robustoides*	Oder river (Gryfino, Poland)	23-Jun-15	**4**	Connective, tegument and muscle	Absent	Connective, tegument and muscle	7.39 ± 0.95	–	–
MK913669	*P. robustoides*	Oder river (Gryfino, Poland)	23-Jun-15	**3**	Mainly connective, especially around intestine, gonads and hepatopancreas. Tegument	Few unicellular stages, mainly in tegument	Connective and tegument	7.03 ± 1.05	Not measurable	Not measurable
MK913665	*G. varsoviensis*	Poreba Kocewy (Poland)	21-Jun-15	**–**	–	–	–	–	–	–
MK913663	*E. marinus*	Newton's Cove (UK)	08-Jun-16	**3**	Mainly connective, especially around intestine, gonads and hepatopancreas. Tegument and haemolymph	Haemolymph, connective, and tegument	Mainly connective	6.27 ± 0.81	LENGTH: 2.43 ± 0.24 WIDTH: 1.72 ± 0.19	LENGTH: 4.08 ± 0.55 WIDTH: 1.36 ± 0.16
MK913662	*E. marinus*	Newton's Cove (UK)	08-Jun-16	**3**	Connective, tegument, hepatopancreas and haemolymph (inside haemocytes)	Haemolymph and tegument	Connective and tegument	7.08 ± 1.08	LENGTH: 2.63 ± 0.42 WIDTH: 1.87 ± 0.28	LENGTH: 4.14 ± 0.45 WIDTH: 1.40 ± 0.16
MK913655	*E. marinus*	Newton's Cove (UK)	13-Sep-16	**1**	Connective, especially around intestine and hepatopancreas. Few plasmodia in tegument	Absent	Mainly connective. Tegument	6.32 ± 0.70	–	–
MK913657	*Gammarus* sp.	Newton's Cove (UK)	14-Dec-16	**3**	Connective around intestine and less in tegument. Muscle also infected	Haemolymph and tegument	Connective	7.64 ± 1.31	LENGTH: 2.49 ± 0.27 WIDTH: 1.66 ± 0.16	Absent
MK913671	*E. marinus*	Newton's Cove (UK)	20-Apr-16	**1**	–	–	–	–	–	–
MK913656	*Orchestia* sp.	Butrón estuary (Spain)	30-Aug-17	**4**	Mainly haemolymph. Connective, tegument and muscle.	Many uninucleated and binucleated stages in haemolymph	Few plasmodia. Connective	8.36 ± 1.28	LENGTH: 3.59 ± 0.22 WIDTH: 2.04 ± 0.22	LENGTH: 3.86 ± 0.40 WIDTH: 3.18 ± 0.33
MK913666	*Orchestia* sp.	Butrón estuary (Spain)	30-Aug-17	**4**	Mainly haemolymph. Connective and tegument.	Haemolymph and tegument.	Connective around intestine and gonads. Tegument	8.13 ± 1.12	LENGTH: 3.65 ± 0.38 WIDTH: 2.09 ± 0.19	LENGTH: 3.61 ± 0.35 WIDTH: 3.12 ± 0.32
MK913668	*Orchestia* sp.	Dart estuary (UK)	27-Apr-17	**3**	Connective, tegument and haemolymph	Haemolymph and tegument.	Connective around intestine and gonads. Tegument	9.24 ± 1.04	LENGTH: 3.63 ± 0.24 WIDTH: 2.00 ± 0.23	LENGTH: 3.80 ± 0.31 WIDTH: 3.13 ± 0.34
MK913667	*Orchestia* sp.	Dart estuary (UK)	27-Apr-17	**4**	Connective completly substituted. Tegument and haemolymph	Haemolymph and tegument.	Connective around intestine and gonads.	9.59 ± 1.10	LENGTH: 3.49 ± 0.42 WIDTH: 2.15 ± 0.22	LENGTH: 4.22 ± 0.32 WIDTH: 3.04 ± 0.37

Paired T test comparing the length of monokaryotic cells of *E. marinus* and *Orchestia* sp. gives a p-value < 2.2 e^{-16}. Mann Whitney U test for the width of monokaryotic cells (not normal distribution): p-value < 2.2 e^{-16}. Paired T test for the length of dikaryotic cells: p-value = 1.48 e^{-07}. Paired T test for the width of dikaryotic cells: p-value < 2.2 e^{-16}.

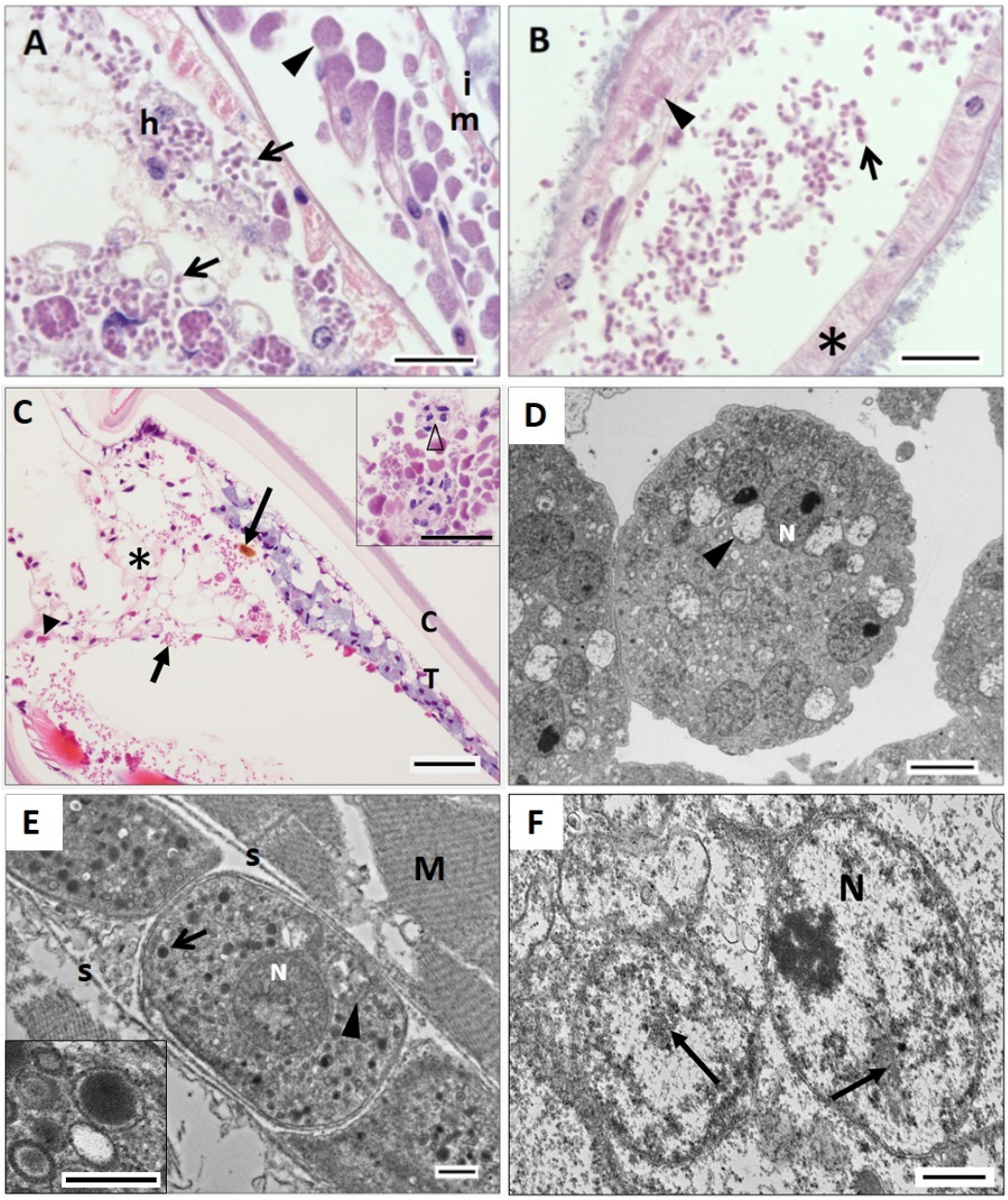

Fig. 2. Histological appearance and ultrastructure of *Haplosporidium echinogammari* infecting host individual MK913663 (*Echinogammarus marinus*, sampled in Newton's Cove the 8th June 2016). **(A)** Unicellular stages of the parasite found loose in the haemolymph (arrows) and within haemocytes (h). Plasmodial stages (arrowhead) filling the gap between the muscle layer (m) around the intestine (i) and the cardiac tissue. **(B)** Unicellular stages of the parasite (monokaryotic and dikaryotic) within the haemal space (arrow) bounded by gill epithelium (*), also plasmodial stages (arrowhead) disrupting a gill epithelium cell. **(C)** Host mediated response to parasite infection includes melanization (long arrow) and granuloma formation (transparent arrowhead in the insertion). Host tissue disruption associated with unicellular stages (short arrow) and plasmodia (arrowhead) is patent in the connective (*) and tegument (t) which is associated with the carapace (c). **(D)** Transmission electron micrograph of a plasmodial stage of the parasite with at least 5 clear nuclei (N) and mitochondria (arrowhead). **(E)** Transmission electron micrograph of a unicellular stage of the parasite enveloped by host sarcolemma (s), interfering and substituting muscle fibres (M). A well-defined single nucleus (N), mitochondria (arrowhead) and haplosporosome-like structures (arrow and insertion) can be observed. **(F)** Transmission electron micrograph of two ovoid electro-lucent nuclei (N) with peripheral compact chromatin, and microtubules (arrows). Scale bars A and B = 20 µm, C = 50 µm (insertion = 50 µm), D = 2 µm, E = 500 nm (insertion = 250 µm), F = 500 nm.

In contrast, plasmodia of the parasite group infecting *Orchestia* sp. amphipods were more irregular and showed large nuclei with dispersed chromatin, and fewer mitochondria (Fig. 3C). Similarly, monokaryotic forms in *E. marinus* (Fig. 2E) possessed a central spherical nucleus, in an electron-lucent cytoplasm in which few clear mitochondria, electron dense vesicles (DVs), haplosporosome-like bodies (HLBs) and vesicles with two concentric membranes were observed (Fig. 2E). Unicellular cells infecting *Orchestia* sp. had an irregular central nucleus with the chromatin widely distributed within a large amorph nucleolus (Fig. 3C). No apparent mitochondria, and fewer haplosporosome-like bodies and double membrane vesicles in a more electron dense cytoplasm (Fig. 3D) were observed.

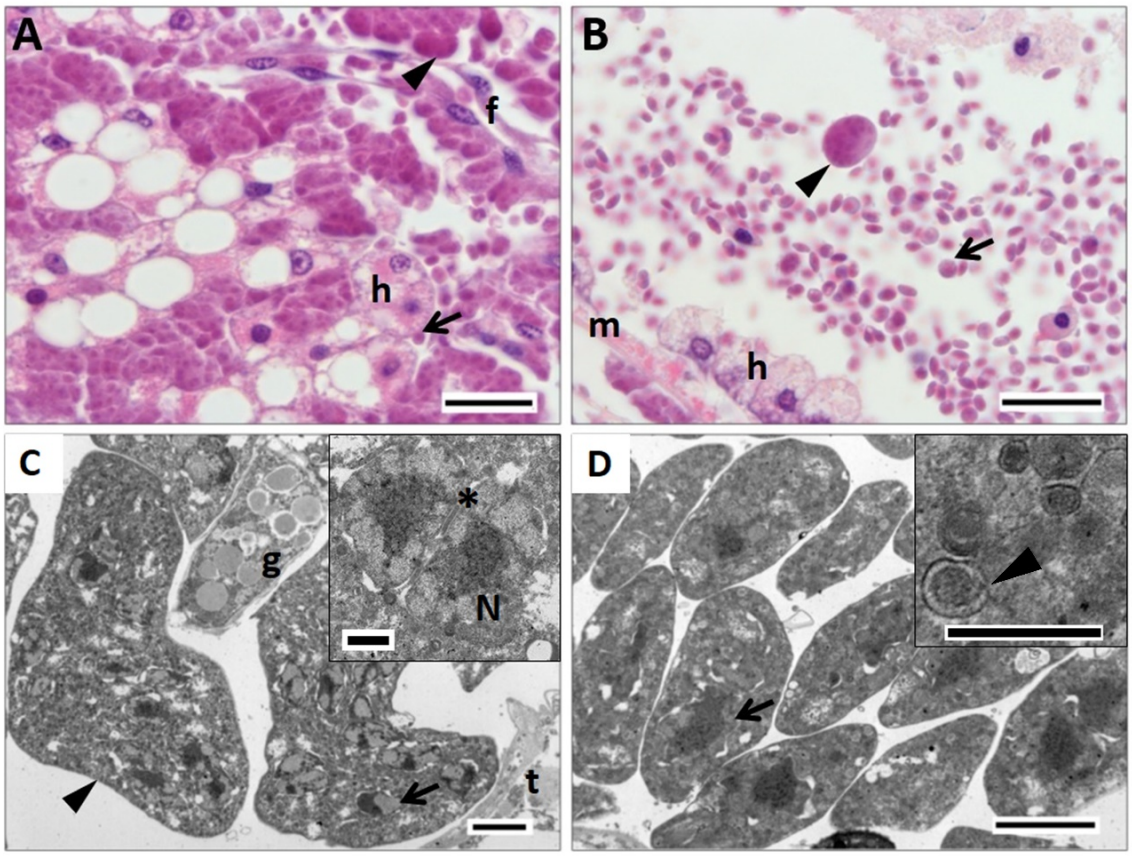

Fig. 3. Histological appearance and ultrastructure of *Haplosporidium orchestiae* infecting amphipod host individual MK913668 (*Orchestia* sp. sampled in Dart estuary (UK) the 27th of April 2017). **(A)** Congestion of the connective tissue around fibroblasts (f) and haemocytes (h), with plasmodial (arrowhead) and unicellular stages (arrow) of the parasite. **(B)** Monokaryotic and dikaryotic unicellular stages (arrow) and a single plasmodia (arrowhead) crowding the haemal sinus and sometimes within haemocytes (h) associated with the cardiac musculature (m). **(C)** Transmission electron micrograph of two multinucleated plasmodia (arrowhead) within the haemal sinus of the host. Also, a host granulocyte (g) and a fraction of disrupted tegument (t) can be observed. Uneven nuclei with sparse chromatin (arrow) separated by an electron-dense cytokinetic structure (*) can be observed in the insertion. **(D)** Transmission electron micrograph of unicellular stages of the parasite with one or two nuclei (arrow). Detail of haplosporosome-like bodies (arrowhead) shown in the insertion. Scale bar: A and B = 20 µm, C and D = 2 µm and both insertions = 500 nm.

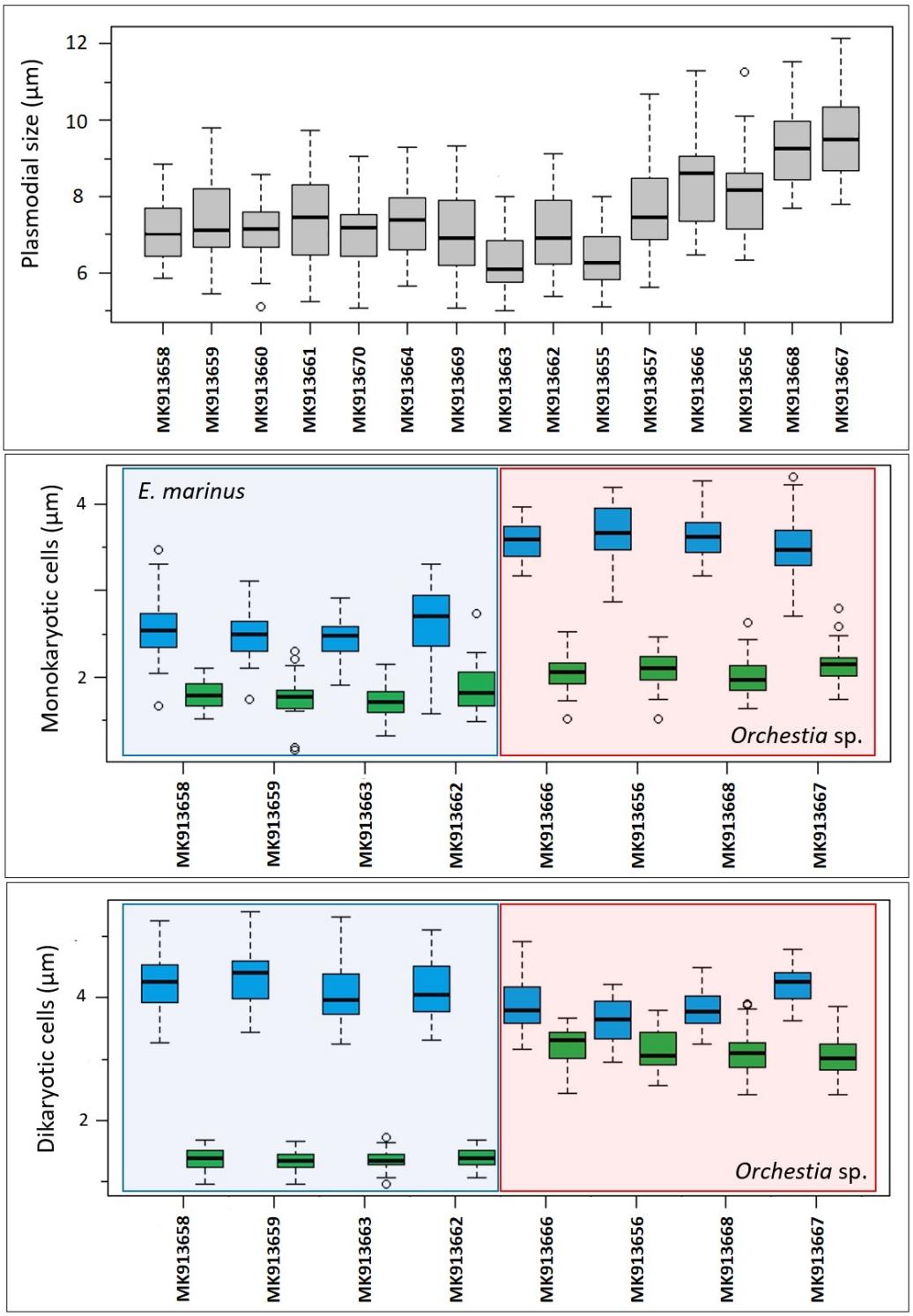

Fig. 4. Box plot for plasmodial and unicellular stages of each haplosporidian parasite (Y axis) against the host infected (X axis). Top graph shows the median, 1st and 3rd quartiles, minimum and maximum (µm) of plasmodial stages for 15 hosts (reference information in Table 3). Middle graph compares the length (blue boxes) and width (green boxes) for monokaryotic unicellular stages, when present and measurable. Bottom graph displays length (blue box) and width (green box) for dikaryotic unicellular stages when present and measurable.

3.3 Molecular phylogeny

Haplosporidian SSU sequences (c. 650 bp long, including the V9 region) were generated from 17 individual amphipods (Fig. 5). Clades I and II contain sequences from *H. orchestiae* and *H. echinogammari,* respectively. Clade III was phylogenetically distinct from clade II, but was closely related to it.

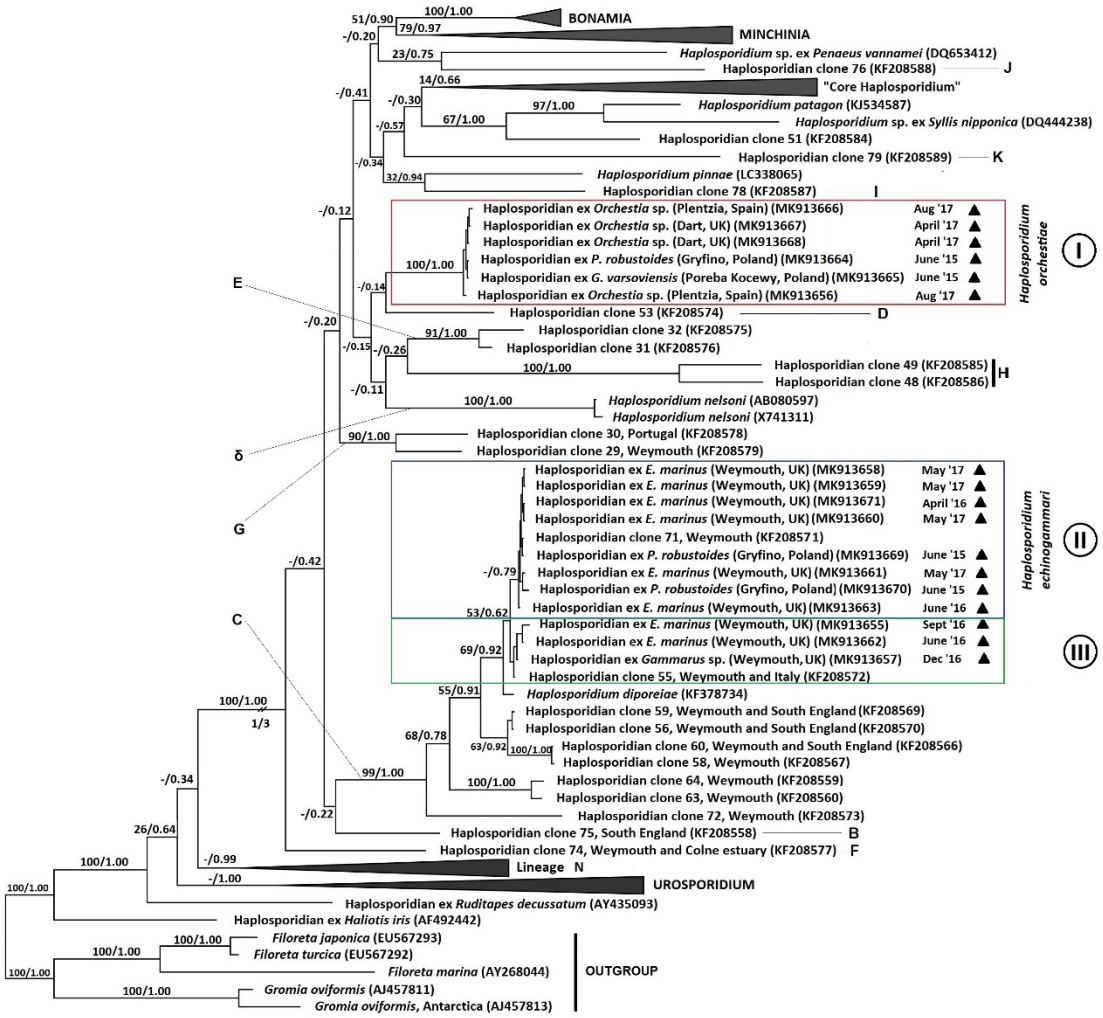

Fig. 5. Two novel haplosporidian species, H. orchestiae and H. echinogammari isolated from different amphipod species, in different locations, form two separate clades on the phylogenetic tree of Haplosporidia. The consensus phylogenetic tree of the order was generated using Bayesian inference. Posterior probabilities and bootstrap support from the Maximum Likelihood (ML) analysis are indicated in the branches. A triangular marker to the right of the sequence reference indicates those samples sequenced by this study. Reference numbers from GenBank are shown to the right of the parasite species or haplosporidian clone number. A roman number inside a circle indicates the three clades commented in the results section. Sequences belonging to genus Bonamia sp., Minchinia sp., "Core" Haplosporidium sp., Urosporidium sp. and haplosporidian lineage "N" associated with Urosporidium sp. have been collapsed. Outgroup includes the same 5 species of ascetosporeans included in (Hartikainen et al. 2014).

BlastN searches against GenBank revealed that sequences comprising the novel species *H. echinogammari* (Clade II) and Clade III were 97-98 % similar to *H. diporeiae* (KF378734; 68 % coverage) and 97-99 % similar to environmental sequences KF208571 and KF208572 corresponding to haplosporidian clones 71 and 55 respectively (from the water column and sediment samples from Weymouth and Italy). The closest BlastN matches to *H. orchestiae* sequences were 93 % - 94 % similar (86 % coverage) to uncultured Haplosporidian clone 29 (KF208579). However, Fig. 5 shows clone 53 (KF208574), sampled from the brackish waters of the fleet and marine waters of Newton's Cove, as the phylogenetically closest previously known relative of *H. orchestiae,* but there is no ML bootstrap or negligible Bayesian Posterior Probability support for the relationship. Although the branching position of *H. echinogammari* within lineage C (Hartikainen et al. 2014) is strongly supported, *H. orchestiae* is not strongly related to any previously known *Haplosporidium* lineage. The Polish freshwater amphipods were infected with both new *Haplosporidium* species. *H. orchestiae* infecting *G. varsoviensis* (MK913665) was 99.6 % similar to that infecting *P. robustoides* (MK913664), and *H. echinogammari* infecting *P. robustoides* (MK913670 and MK913669) were 99.1 % and 99 % similar to (MK913661), from Weymouth. Within *H. echinogammari*, all amphipod-derived haplosporidian sequences were sampled from Newton's Cove between April and June in 2016 and 2017. MK913655 and MK913657, in Clade III, were collected in the same location in September and December 2016 respectively. *Orchestia* sp. was only observed and collected in Newton's Cove in September, but no haplosporidian infections were detected.

Phylogenetic analysis including sequences of haplosporidians amplified from other amphipods, isopods, crabs and crayfish (Fig. 6), none of which had matching histological or ultrastructural observations, showed that 22 out of 25 of these crustacean-derived sequences grouped within three main clades (Fig. 6). The reference codes and provenances of these sequences are shown in Table 4. Clade 1 included haplosporidians isolated from *Cancer pagurus* and *Carcinus maenas* incubations in artificial seawater, but also parasite infected tissues from a crab sampled in Thailand, and from freshwater amphipods sampled in Florida and UK. This clade also included lineages E and G from (Hartikainen et al. 2014), the notorious oyster parasite *H. nelsoni*, and *H. orchestiae*. However, there was negligible support for the whole clade. Clade 2 had less than 50 % ML bootstrap and 0.92 Bayesian PP support, but comprised two more strongly supported clades, which corresponded to lineages F and B in Hartikainen et al. (2014), which did not branch together in the latter analysis. Prior to this study, both B and F were represented by only one sequence each, B from a freshwater column, and F from Fleet water (Weymouth). Seven of our sequences, from isopods, crayfish, crabs, and amphipods, grouped

with B, and three (from amphipods and an isopod) grouped with F, all from brackish or freshwater sites. No non-crustacean hosts are currently known from Clade 2. Clade 3 (97/1.00), corresponding to lineage C from Hartikainen et al. (2014), contained nine novel sequence types generated by this study, six associated with crabs, and the rest with amphipods. In addition, this clade included *H. echinogammari* and *H. diporeiae*. All other previously known sequence types in this clade were either crustacean-associated or from water or sediment samples.

Table 4. Novel haplosporidian sequences obtained from parasites associated with crustacean hosts included in Fig. 6. The reference used for each sequence in the phylogenetic tree (Fig. 6) is related to the host species if known, the sampling location and year of collection, and whether the sample was obtained from infected tissue or from the filter after incubation in artificial seawater.

Crustacean taxa	Sequence reference	Host species	Sampling location	Tissue infection / Incubation water
Order Amphipoda	Amphipod seq. 1	Unknown	St. John river (Florida), Leeds (UK)	Tissue
	Amphipod seq. 2	Unknown	Newton's Cove (UK)	Tissue
	Amphipod seq. 3	*Gammarus* sp.	River Avon (UK)	Tissue
	Amphipod seq. 4	Unknown	Fresh water environment (UK)	Tissue
	Amphipod seq. 5	Unknown	Fresh water environment (UK)	Tissue
	Amphipod seq. 6	Unknown	St. John river (Florida)	Tissue
	Amphipod seq. 7	Unknown	Fresh water environment (UK)	Tissue
Order Isopoda	Isopod seq. 1	*Asellus* sp.	River Avon (UK)	Tissue
	Isopod seq. 2	*Asellus* sp.	River Itchen (UK)	Tissue
	Isopod seq. 3	*Asellus* sp.	River Itchen (UK)	Tissue
Order Decapoda	Crab seq. 1	Unknown	Fresh water environment (Thailand)	Tissue
	Crab seq. 2	*C. pagurus* and *C. maenas*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 3	*C. pagurus* and *C. maenas*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 4	*Carcinus maenas*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 5	*C. pagurus* and *C. maenas*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 6	*C. pagurus* and *C. maenas*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 7	*Cancer pagurus*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 8	*Cancer pagurus*	Newton's Cove (UK) 2013	Incubation water
	Crab seq. 9	*Eriochier sinensis*	River Thames, 2014	Incubation water
	Crab seq. 10	*Eriochier sinensis*	River Thames, 2014	Incubation water
	Crab seq. 11	*Cancer pagurus*	Newton's Cove (UK), 2013	Incubation water
	Crab seq. 12	*Cancer pagurus*	Newton's Cove (UK), 2013	Incubation water
	Crayfish seq. 1	*Procambarus clarkii*	Hampstead Head, Pond (UK), 2015	Tissue
	Crayfish seq. 2	*Procambarus clarkii*	Hampstead Head, Pond (UK), 2015	Tissue

Only a few haplosporidian sequences from crustacean hosts branched outside of these clades. Haplosporidian sequence (Crab seq. 9) obtained from the incubation water of an individual of *C. pagurus* from Newton's Cove, branched robustly (87/0.99) as sister to *Urosporidium*, and was very similar to KF208597 from Newton's Cove water column (clone 90). Crab seq. 3, also from Newton's Cove *C. pagurus* incubation water, robustly branched within lineage N (97/1.00). The only haplosporidian sequence generated from amphipod tissues that does not group in any of the three described crustacean clades, was Amphipod seq. 6 from St John's River (Florida), which was sister to *H. pickfordi*, a parasite of freshwater snails, in the "core" *Haplosporidium* clade.

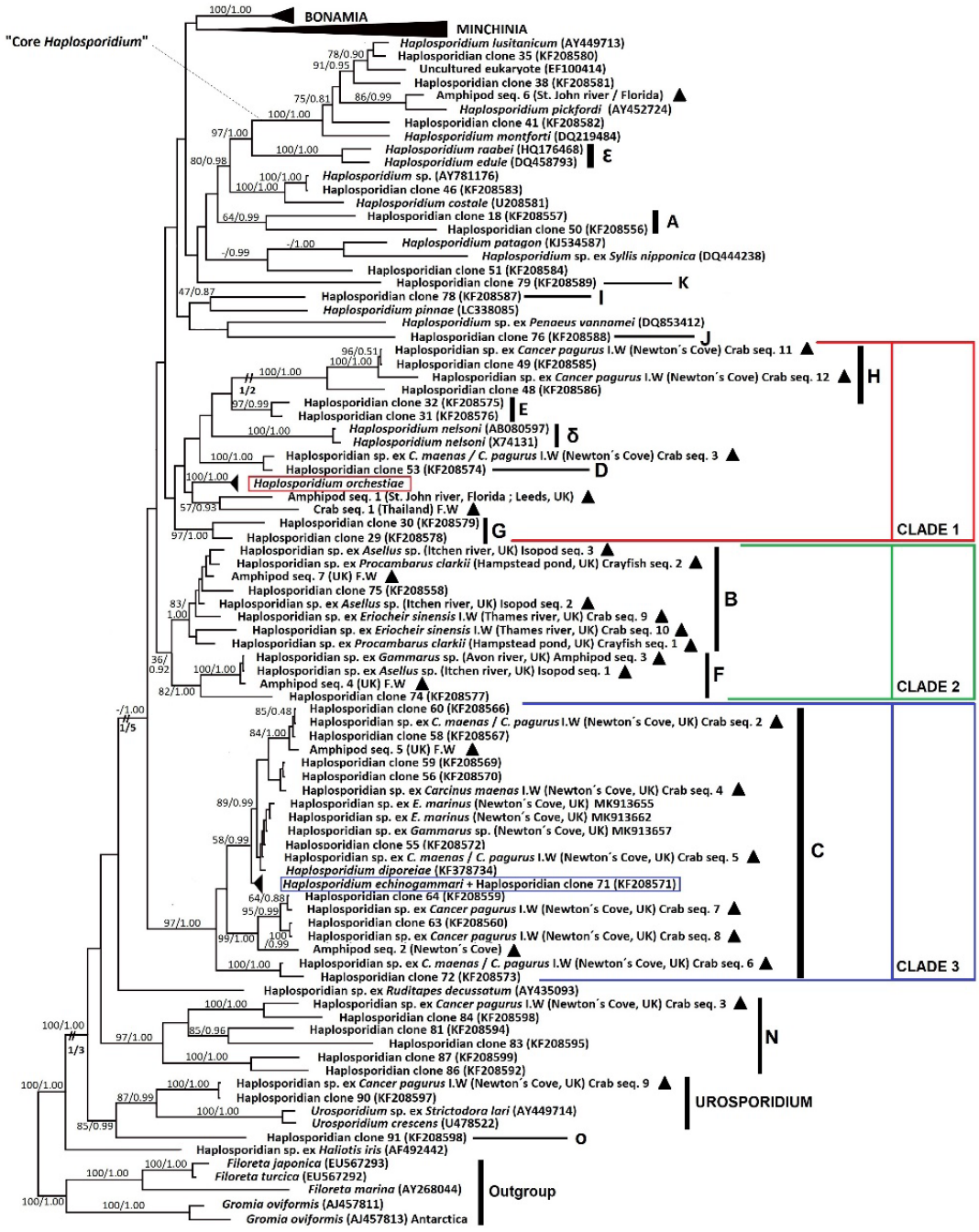

Fig. 6. The phylogenetic analysis demonstrates that most haplosporidian sequences associated with crustacean hosts, group together in three clades (Clade 1, 2 and 3 on the figure). The consensus phylogenetic tree of the order Haplosporida was generated using Bayesian inference. Nodes support values of <75 % (ML bootstrap) and <0.85 (Bayesian Posterior Probability) are not shown. Where nodes are supported above either threshold, both values are given. The triangular marker to the right of the sequence reference indicates sequences associated with crustacean hosts included by the present study besides those from *H. echinogammari* and *H. orchestiae*. Reference numbers from GenBank are specified to the right of the parasite species or haplosporidian clone number. Sequences of the genus *Bonamia* sp., and *Minchinia* sp. and groups of sequences forming *H. echinogammari* (blue rectangle) and *H. orchestiae* (red rectangle) previously included in Fig. 5 have been collapsed. Lineages established in (Hartikainen et al. 2014) are indicated with letters (A to O).

3.4 Taxonomic summary

Novel species within existing lineage C (Hartikainen et al. 2014)

- Phylum: Endomyxa Cavalier-Smith, 2018
- Class: Ascetosporea Cavalier-Smith, 2002
- Order: Haplosporida Caullery & Mesnil, 1899
- Family: Haplosporiidae Sprague, 1979
- Genus: *Haplosporidium* Caullery & Mesnil, 1899
- Species: ***Haplosporidium echinogammari*** **sp. nov. Urrutia, Feist and Bass, 2018**
- Diagnosis: Spherical to elongated monokaryotic stages, with a length varying between 2.6 and 2.9 µm and a width around 1.7 ± 0.2 µm, they develop a more tubular shape when dikaryotic (length 4.5 ± 0.4 µm; width: 1.25 ± 0.2 µm). No sporulation observed. The plasmodia range in size between 6.6 µm and 8.02 µm in diameter with no more than 20 nuclei/section. Infection develops in the connective tissue associated with digestive and tegumental glands, from where it spreads to other organs, eventually producing tissue disruption.
- Type host: Amphipods. *Echinogammarus marinus*
- Type location: Coastal waters in Newton's Cove (UK).
- Type material: Original slides used for this paper are stored together with biological material embedded in wax and epoxy resin in CEFAS Weymouth Lab. The type material is stored as RA 16046 (specimen n° 19), and the SSU rDNA sequence deposited in GenBank with the reference: MK913663.

Novel species within novel lineage

- Phylum: Endomyxa Cavalier-Smith, 2018
- Class: Ascetosporea Cavalier-Smith, 2002
- Order: Haplosporida Caullery & Mesnil, 1899
- Family: Haplosporiidae Sprague, 1979
- Genus: *Haplosporidium* Caullery & Mesnil, 1899
- Species: ***Haplosporidium orchestiae*** **sp. nov. Urrutia, Feist and Bass, 2018**
- Diagnosis: Spherical to elongated monokaryotic stages, with a length between 3.3 µm and 3.7 µm and a width around 2.2 ± 0.25 µm, become subspherical when dikaryotic (length 3.8 ± 0.3 µm; width: 3.2 ± 0.2 µm). No sporulation observed. The plasmodia range in size between 7.81 µm and 9.4 µm in diameter with no more than 15 nuclei/section. Infection

develops in the connective tissue associated with digestive and tegumental glands, from where it spreads to other organs, eventually producing tissue disruption.

- Type host: Amphipods. *Orchestia* sp.
- Type location: Estuarine waters in Dart (UK).
- Type material: Original slides used for this paper are stored together with biological material embedded in wax and epoxy resin in CEFAS Weymouth Lab. The type material is stored as RA17028 (specimen n° 47), and the SSU rDNA sequence is deposited in GenBank with the reference: MK913668.

4. Discussion

Despite their ecological and economic importance, members of the order Haplosporida are understudied. The taxonomic relationships within the group remain a challenge more than a century after the discovery of the first species. Ecology, geographic distribution and biological cycle of most described species are poorly understood or unknown. The idea of a highly diverse order Haplosporida is not new. Previous phylogenetic studies (Reece et al. 2004, Hartikainen et al. 2014) have shown significant genetic diversity within the group and the likely paraphyly of the most species-rich genus, *Haplosporidium*. Hartikainen et al. (2014), published sequences (SSU rDNA) of 85 lineages, but only 48 % of them have a known or suggested host. 75 % of the remaining haplosporidian sequences, including 14 novel highly divergent lineages, were from environmental samples (filtered water and/or sediment). Moreover, there is a need for host-based field surveys in order to characterize those clades and the species constituting them (Hartikainen et al. 2014). Our histopathological survey of prominent invertebrate groups in coastal ecosystems in the south of England, showed amphipods to be frequently infected by haplosporidian parasites. While *H. orchestiae* sequence type didn't match with any of the environmental sequences in Hartikainen et al. (2014), *H. echinogammari* type sequence (MK913663) was almost identical to KF208571 collected from the water column in Newton's Cove. Moreover, it was closely related to KF208572 and other sequences within Lineage C (KF208566, KF208567, KF208569, KF208570) found in the water column and the sediment in the same location.

Morphological discrimination of haplosporidians is often based on characterization of the spore ornamentation, size, and ontogeny of the spore (Perkins 2000, Burreson & Reece 2006). Interestingly, in contrast to sporogenesis of *Haplosporidium diporeiae* in the amphipod genus *Diporeia*, which was observed to occur synchronously with continued plasmodial

development (Messick 2009, Winters & Faisal 2014), we detected no sporogonic stages or mature spores in our samples (n=64), despite sampling over two infection cycles of *H. echinogammari* at least, and our samples representing a range of infection stages. The absence of spores in our samples may have (one of) several explanations, including sporogenesis occurring only under certain conditions that were not met in our samples, the infection cycle completing without spores being produced, sporogenesis taking place in other host species, or simply be because our sampling missed the sporulating stages. However, efforts to find alternate hosts in the life cycle of *H. echinogammari* and *H. orchestiae* have been unsuccessful. The fate of spores after they are released from the host remains unknown, even for the best studied haplosporidian, *H. nelsoni* (Carnegie & Burreson 2012). In *H. orchestiae*, and *H. echinogammari* initial infection is observed in tissues directly exposed to the environment, like tegument and gills, but also in the connective around the intestine, leaving open several possibilities as the portal of infection.

Description of spore morphology is not an essential requirement for the description of protistan parasite taxa when other phenotypic and phylogenetic characteristics are considered together. In the case of the two new species described here, morphological features of the plasmodial and unicellular stages present in *Orchestia* sp. and *Echinogammarus* sp. were sufficient to differentiate the parasites. Morphological, including ultrastructural, and phylogenetic differences between *H. orchestiae* and *H. echinogammari* clearly define them as distinct taxa. The phylogenetic relationships of *H. orchestiae* to existing uncharacterized lineages (D, E, H, and δ) described in Hartikainen et al. (2014) are unresolved, and it is clearly distinct from the apparently closest related characterized species, *H. nelsoni* (Haskin et al. 1966). *H. echinogammari* is more closely related to a previously characterised species, *H. diporeiae*, but is distinguished from it phylogenetically, and perhaps by spore-forming propensity. There are insufficient data available to determine whether *H. echinogammari* can be distinguished from *H. diporeiae* on the basis of ultrastructure or histological appearance.

Since the evolutionary distance between Haplosporidian clone 71 (KF208571), collected from Weymouth in 2012 (Hartikainen et al. 2014) and *H. echinogammari* was smaller than the distance between some of the sequences within the clade, clone 71 was reassigned as *H. echinogammari*. Not all the haplosporidians infecting *E. marinus* are classified as *H. echinogammari*. Clade III on Fig. 5, which includes parasites sampled from the same host and location (Newton's Cove), is formed by two haplosporidians infecting *Gammarus* and *Echinogammarus*, plus Haplosporidian clone 55 (KF208572) isolated from Newton's Cove and a freshwater system in Italy. Clade III is phylogenetically distinct from clade II, but because

morphology and histopathology of the parasites examined from clade III were indistinguishable from those of clade II, and no ultrastructural data are available for clade III, there is insufficient evidence to classify clade III as either *H. echinogammari*, or a different novel species. Future work based on larger sample numbers may justify its separate description. We note that, in contrast to *H. echinogammari* which appears to infect *E. marinus* in late spring (May and June), this second group of parasites are responsible for infections developed during more varied times of the year suggesting a potential role for seasonality in discriminating between these lineages.

Only two further amphipod species have previously been found to harbour haplosporidian infections: *Rivulogammarus pulex* and *Parhyale hawaiensis*, the first from freshwater systems in Belgium and Sweden (Van Ryckeghem 1930, Larsson 1987), and the second in the Egyptian coast of the Red Sea (Ismail 2011). No genetic sequence data are available for either parasite and ultrastructural comparison is difficult due to the lack of similar stages presented in the studies. Lineage C (Hartikainen et al. 2014) was presented as a highly diverse group with a clear preference for estuarine and rocky shore locations. While 40% of the sequences within the clade are currently uncharacterized, the remainder derives from different species of amphipods sampled from diverse aquatic environments. We suggest that evolutionary diversification of haplosporidians within lineage C might in future be related to amphipod host specificity and/or seasonality, a factor feature proposed for other parasite groups (Lange et al. 2015, González-Tortuero et al. 2016).

While molluscs may be the most diverse hosts for haplosporidian parasites, crustaceans are increasingly reported as hosts (Stentiford et al. 2013), including infections affecting commercial species (Bower & Meyer 2002, Utari et al. 2012). However, crustacean-derived haplosporidians have so far appeared to branch without discernible pattern among mollusc-infecting lineages. For instance, *H. littoralis* infecting crabs is included within the "core" haplosporidium group, which includes well-known parasites of oysters, mussels, cockles and snails (*H. costale*, *H. pickfordi*, *H. edule*, *H. montforti*, *H. raabei*), while the haplosporidian infecting shrimp *Pennaeus vannamei* (Dyková et al. 1988, Nunan et al. 2007) is related to uncharacterized lineages F and G, *H. diporeiae* to the recently characterized amphipod-infecting lineage C, and *H. louisiana* is close to trematode-infecting *Urosporidium* sp. The phylogenetic analyses in this paper include 25 novel sequences of haplosporidians either infecting or associated with crustacean hosts, 92 % of which group together in three crustacean-rich clades. Clade 1 includes *H. nelsoni* and lineages D, E, G, and H, originally defined in Hartikainen et al. (2014), to which this study adds *H. orchestiae*. Support for this clade is extremely weak, although it contains several strongly supported clades: the long-branched H is so far only associated with

crustaceans as putative hosts, as is D, and novel lineages detected in this study (Fig. 6). The haplosporidians detected in crab incubation water experiments suggest that crabs are hosts or/and vectors of these parasites.

In clade 2 we show that lineages B and F in Hartikainen et al. (2014) are also dominated by crustacean-associated haplosporidians from brackish and freshwater habitats. No mollusc-associated haplosporidians have yet been detected in these clades, although non-marine mollusc hosts have not been sampled anywhere near as intensively as our screening of potential crustacean hosts in this study, and marine molluscs previously. Of additional interest is our finding that, with the increased taxon sampling from the present study, lineages B and F are grouping together, albeit relatively weakly, which was not the case in Hartikainen et al. (2014). In clade 3 (lineage C of Hartikainen et al. 2014), amphipod-derived parasite lineages are often closely related to those amplified from crabs. This might indicate some correspondence between crab and amphipod parasites. Perhaps their life cycles involve both hosts, there are relatively easy evolutionary transitions between crab and amphipods as main hosts, or their detection in crabs derives from infected amphipods being consumed by crabs. A dedicated investigation of crab-infecting haplosporidians at the same site would clarify this. However, it is noteworthy that the two new *Haplosporidium* species described in this paper have only ever been amplified from amphipod tissue or, and only in the case of *H. echinogammari*, host-independent environmental samples.

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GENERAL DISCUSSION

GENERAL DISCUSSION

The unremitting expansion of molecular biology has been attracting researchers from multiple disciplines to the study of protists, a world traditionally circumvented due to microscopic size, quick interactions and ambiguous taxonomy (Cavalier-Smith 1995, Adl et al. 2007, Gao et al. 2019, Keeling & Burki 2019). Accordingly, the significance of the role played by unicellular eukaryotes in aquatic ecosystems as autotrophs, heterotrophs, and myxotrophs appears to be increasing by the day (Mitra et al. 2014, Bjorbækmo et al. 2020). The revolution instigated by Next Generation Sequencing (NGS) methods in the analysis of environmental and organismal matrixes (eDNA), is opening "Pandora´s Box" of micro-eukaryotic diversity (Massana et al. 2015, Bass et al. 2015, Burki et al. 2020). The discovery of such a vast amount of **cryptic species** goes beyond the simple escalation in biodiversity; it affects studies on cell-biology, ecology, lifecycle, and spatiotemporal distribution among others (Poulin 2014, Oliverio et al. 2020).

The extent of this **hidden diversity** is particularly significant among several lineages of **protist parasites** (de Vargas et al. 2015, Tashyreva et al. 2018, Schoenle et al. 2021). Such lineages are often constituted by morphologically-reduced and phenotypically-convergent species (Perkins et al. 2011) that make challenging microscopy-based discrimination between taxa (Martinsen et al. 2007). As a result, there is a sizeable underestimation in the number of existing species within many parasite-rich protists lineages, in which the number of phylospecies is still rising steeply or just beginning to show a slow down (Caron & Hu 2019). In fact, some recent and wide-ranging environmental surveys show parasitism as a predominant consumer strategy among aquatic micro-eukaryotes (Skovgaard 2014, de Vargas et al. 2015, Jephcott et al. 2016). These results support earlier mathematical models suggesting that parasites represent at least half of the species on earth (Windsor 1998, de Meeûs & Renaud 2002, Dobson et al. 2008).

In light of these findings, the generalized conception of parasites being little else than a "nuisance" for humans and their interests, is steadily shifting into recognition of their indispensable role in the environment (Lafferty et al. 2006, Rigaud et al. 2010). Parasites are key players in host population dynamics, holding back disproportionate growth of common species, and increasing diversity (Dunne et al. 2013). Therefore, parasite-host interactions should not only be studied for their potential to cause disease in species of human concern, but also as an indispensable part of the food-web and the ecosystem (Hudson et al. 2006, Buck 2019). Actually, ecological models set to understand biological interactions and energy flows in the ecosystem

have seen far-reaching variations after considering parasitic links (Lafferty et al. 2008, Dunne et al. 2013, Preston et al. 2014).

Limited insight on the vast majority of parasite-host interactions, as epitomized by the enormous hidden diversity being revealed, is a major obstacle in the study of parasites either as human-concerning pathogens (human disease, aquaculture, zoonoses), or key elements of the ecosystem. In this context, there is certainly a firm interest in finding those cryptic species and associating them to a morphology and ultrastructure, whilst describing their cell-biology, pathology, host-range, prevalence, or spatiotemporal distribution (Nadler & Pérez-Ponce de León 2011, Kmentová et al. 2016, Galipaud et al. 2019). Furthermore, this appeal can be made extensive to already described protists parasite species, many of which have complex lifecycles that remain largely unsolved (Bass et al. 2015, Okamura 2016, Blasco-Costa & Poulin 2017).

In this framework, this thesis hypothesized that common invertebrate species in the macrobenthos; a pivotal but under-screened component of the food-web in marine ecosystems (Marcogliese 2002, Lobo et al. 2017), could host a significant fraction of that hidden diversity. Additionally, it assumed the task of describing that diversity morphologically, histopathologically, and molecularly; setting up the base for a subsequent monitoring of its variability through space and time. Histopathology was chosen as screening method for this baseline analysis, in order to analyze spatiotemporal variability of protist-caused **infection**; in contraposition to parasite **presence,** frequently analysed by molecular-based studies (Valkiūnas 2011, Aranguren & Figueras 2016, Burge et al. 2016, Sato et al. 2019).

In order to test this hypothesis, an ecological analysis of the intertidal zone was conducted in Newton´s Cove (Weymouth), a temperate coastal location in South British Isles (**Chapter 1**). This rocky beach, characterized by an ample tidal range, continuous vertical profile,and heterogeneity of features (rocks, pools, ridges, fissure), has been shown to horde great hidden protist diversity in past environmental surveys (Hartikainen et al. 2014a, Hartikainen et al. 2014b, Hartikainen et al. 2016, Ward et al. 2016, Ward et al. 2018). Moreover, a significant number of novel protist parasites with uncharacterized lifecycles have been described in this location (Feist et al. 2009, Bateman et al. 2011, Stentiford et al. 2013). As parasite prevalence is often constrained (not exclusively) by host population density and position in the intertidal zone (Ebert 1995, Choisy et al. 2003), the ecological analysis of Newton´s Cove aimed not only to find common and ecologically relevant species, but also to place them in spatial and community-structural context. The results of the subsequent histopathological screening show that among the most common and abundant taxa:

platyhelminths, annelids, harpacticoid copepods, and amphipods; the later, are particularly susceptible to infection by different lineages of significant protist parasites. However, and despite the extensively demonstrated ecological relevance of amphipods, copepods, polychaetes, and platyhelminths for coastal communities (Martens & Schockaert 1986, Duffy & Hay 2000, Scaps 2002, Turner 2004, Beaugrand & Kirby 2010, Michel et al. 2015), little is known about their associated parasite-communities.

Reviewed in the general introduction (**Section 2**), about a third (27 out of 81) of the eukaryotic lineages (after the eukaryote tree by Keeling & Burki 2019), include invertebrate-infecting species. Excluding multicellular animal and fungal parasites, almost a third of these invertebrate-infecting clades (**endomyxids**, **ciliates**, **apicomplexans**, **syndinians**, **oomycetes**, **microsporidians**) have been identified infecting **amphipods** in southwest England by this study (Chapter 1). Furthermore, the discovery of the amphipod-infecting parasite *Txikispora philomaios* n. sp. (Urrutia et al. 2021) adds **Filasterea** to the list, becoming together with Ichthyosporea the only lineages of protist parasites (*sensu stricto*) within Holozoa, the "animal" branch in late Supergroup Opisthokonta (Glockling et al. 2013, Adl et al. 2019). Additionally, a non-microsporidian fungal-like micro-eukaryote has been microscopically identified parasitizing amphipods in Newton´s Cove (**Chapter 1**). The finding might potentially engross the list of amphipod-infecting protists parasites, but the putative holomycotan (the "fungal" branch within Opisthokonta) must be phylogenetically placed first.

While direct comparison is not advisable due to differences in the sampling effort and non-randomized histopathological screenings, none of the other key invertebrate taxa examined in Newton´s Cove, presented an equivalent level of parasitisation. Highly abundant in Newton´s cove and the British Isles (Warren 1976), the cosmopolitan (Tomioka et al. 2016) polychaete genus *Capitella* was shown to hosts **ciliates**, **apicomplexans**, and **microsporidians**, already known symbionts of this hardly investigated annelid (Larsson & Køie 2006, Rotari et al. 2015). This finding can result, to a certain extent, counterintuitive, as annelids represent the principal hosts for invertebrate-infecting protist parasites (Introduction - Section 2 - Fig. 4) after correction for the clade's size. Similarly, **ciliates** and **gregarines** appeared to be the main protists associated to the turbellarian *Procerodes* sp., although none was observed to cause pathology. In fact, the number of parasitic interactions known within this platyhelminth genus is truly anecdotic, despite the main role of platyhelminths as hosts of protist parasites (Fig. 5; Sokolova & Overstreet 2020). Finally, and in line with findings by Hockin (1984), who investigated the symbionts occurring in harpacticoid populations inhabiting the north of the British Isles, **ciliates** constitute the main protists lineage associated to copepods in Newton´s Cove. This clade of

maxillopod crustaceans, are often hypothesized to be the main reservoir for many lineages of protists parasites (Bass et al. 2021), although, as in this case, often avoid to be confirmed as such.

Identified as important **reservoir hosts** of protist parasites in Newton´s Cove, three amphipod genera (*Echinogammarus*, *Gammarus*, and *Orchestia*) were histopathologically screened from April 2016 to August 2017. This allowed detection of temporal patterns in the infection dynamics of all protist groups, especially among **endomyxid**, **microsporidian**, **filasterean**, and **syndinian** parasites. Additionally, these three amphipod genera were analysed seasonally in four locations in South England, indicating spatiotemporal variability and certain predictability in the occurrence of infections. However, it must be determined first whether these amphipod-infecting parasites correspond to described, cryptic, or novel species.

In the introduction (**Section 4**), the rhizarian clade **Endomyxa** Cavalier-Smith 2002 was discussed to likely accommodate significant hidden diversity. Especially among marine invertebrate-infecting protist lineages, given the predominance of these hosts (61%) among already known species. Four lineages constitute the clade: Vampyrellida, Phytomyxea, Gromiidea, and Ascetosporea, being the later particularly concerning as obligate parasites of invertebrates. Species within the other lineages are either free-living or parasitic for plants, algae, and other protists (Dumack et al. 2020). Class **Ascetosporea** is constituted by five Orders (Haplosporida, Paramyxida, Claustrosporida, Paradiniida, and Mikrocytida), all parasites of invertebrates that hardly share anything else than a complex spore-structure containing one or more sporoplasms (Adl et al. 2019). However, the interest for these long-known pathogens of bivalves such as *Marteilia*, *Bonamia*, *Minchinia*, *Haplosporidium,* or Mikrocytos (Sierra et al. 2016), is bolstered recently by the incorporation to the clade of several orphan lineages of significant parasites and the significnat hidden diversity discovered (Bass et al. 2019). Besides, and despite the great mortality events in commercial and wild bivalve populations (Catanese et al. 2018), the lifecycle of most of this taxa is uncharted, making desirable a better knowledge of their reservoirs and vectors (Reece et al. 2004, Hartikainen et al. 2014a, Mérou et al. 2020).

Two ascetosporean orders, **Haplosporida** Caullery & Mesnil 1899 and **Paramyxida** Chatton 1911, include parasite taxa causing frequent infections in Newton´s Cove amphipod populations, as well as in the rest of coastal locations examined in South British Isles. Morphological discrimination between the two ascetosporeans to the light microscope is feasible, especially when dividing or multinucleated stages are present (**Chapter 1**). While In amphipod-infecting haplosporidians a multinucleated plasmodium (without internal cleavages)

encloses up to 20 identical nuclei (Winters & Faisal 2014, Urrutia et al. 2019), in amphipod-infecting paramyxids the daughter cells (membrane-bound secondary cells) arise by endogeny within a primary cell; forming the characteristic cell within cell structure (Feist et al. 2009). However, and since several species from both clades had been already described parasitizing commercially exploited crab species in this coastal area (Feist et al. 2009, Stentiford et al. 2013, Ward et al. 2016), the two endomyxid parasites were phylogenetically investigated.

The phylogenetic analysis of amphipod-infecting haplosporidians in Newton´s Cove did not correspond to crab-infecting species described in the area, or the British Isles (Stentiford et al. 2013, Davies et al. 2020). They actually constituted two different and highly divergent novel *Haplosporidium* species: *H. echinogammari* n. sp. and *H. orchestiae* n. sp. (Urrutia et al. 2019). While *H. echinogammari* is genetically close to *H. diporeiae*, the only molecularly characterized amphipod-infecting haplosporidian to the date (Winters & Faisal 2014), *H. orchestiae* is more closely related to the notorious bivalve pathogen *Haplosporidium nelsoni,* although constituting a poorly supported clade. Moreover, *H. echinogammari*, is almost identical to uncultured haplosporidian clone KF208571, and very similar to a half a dozen cryptic species isolated from eDNA analyses conducted in Newton´s Cove by Hartikainen et al. (2014a). Given the morphological (light microscopy) similarity between the closely related but distinct *Haplosporidium* spp. infecting genera *Echinogammarus* and *Gammarus*, it is likely that the cryptic species-rich lineage C proposed by Hartikainen et al. (2014a) is constituted in fact by several highly specific amphipod-infecting haplosporidian species (Urrutia et al. 2019)

Therefore, and in view of the fact that a significant amount of haplosporidian hidden diversity might be connected to crustaceans in this traditionally mollusc-infecting clade of pathogens; other amphipod, isopod, crayfish, and crab species from several locations around the British Isles, Spain, Florida, and Poland, were molecularly and histologically searched for haplosporidian parasites (**Chapter 3**). In total, 25 different novel haplosporidian phylospecies have been shown to be either causing infection or associated to crustacean hosts, revealing that crustaceans represent indeed significant but overlooked reservoirs for *Haplosporidium* spp. (Urrutia et al. 2019). Moreover, as yet, the few crustacean-infecting haplosporidians appeared to branch without discernible pattern within the mollusc-infecting lineages (Dyková et al. 1988, Nunan et al. 2007, Utari et al. 2012), however, 92% of these putative crustacean-infecting species have been shown to belong to three crustacean-rich clades. Besides, a handful of the crustacean-derived phylotypes associated to amphipods and crabs were often identical or closely related, suggesting that there might be some correspondence between crab and amphipod parasites. Perhaps their lifecycles involve both hosts; there are relatively easy

evolutionary transitions between crabs and amphipods as main hosts, but further histopathological analysis targeting crabs will be necessary to substantiate this possibility.

Contrasting with this complex of cryptic haplosporidian species, **paramyxids** infecting different amphipod species not only in Newton´s Cove but in southwest England too, appear to have identical 18S rRNA. The sequence and morphological appearance indicate that the paramyxid is *Paramarteilia orchestiae,* histopathologically described by Ginsburger-Vogel & Desportes (1979), but of current interest, given its feminising effect on the host (Pickup & Ironside 2018). The histopathological and phylogenetic analyses by this study expand the known host range for *P. orchestiae*, further supporting the view by Ward et al. (2016) that this species might be less host specific than conceived. In fact, *P. orchestiae* might correspond or be closely related to crab-infecting *P. canceri*, whose 18S rDNA is not sequenced, despite being a pathogen of edible crab (*Cancer pagurus*) throughout the British Isles (Feist et al. 2009, Ward et al. 2016). Until proven the same or different species, both parasites (*P. canceri* and *P. orchestiae*) remain the only crustacean-infecting paramyxid species globally, suggesting hidden diversity among amphipods and crabs in other geographic locations out of the British Isles. In turn, a wider host-range would have significant implications, as the same parasite has been detected in incubation water from *Cerastoderma edule* and *Mytilus edulis* (Ward et al. 2016). Life cycles including bivalve-infecting stages and intermediate crustacean hosts would not be extraordinary within lineage Paramyxida, as copepods have already been demonstrated to be intermediate hosts for *Marteilia refringens*, the causative agent of Aber´s disease in oysters (Audemard et al. 2002, Boyer et al. 2013). Infections of crabs, mussels, or cockles would escalate the significance of *P. orchestiae* as a pathogen of wild and farmed populations highlighting the significance of several amphipod species as common reservoir hosts.

Also part of the TSAR Supergroup, the alveolate lineage **Syndiniales** Loeblich 1976, has been shown to host great hidden diversity (de Vargas et al. 2015, Schoenle et al. 2021), a significant part of which is likely constituted by cryptic parasites of invertebrates (**Introduction - Section 4**). The clade includes few but very significant species of crustacean-infecting parasites, such as *Hematodinium* sp., the causative agent of the “bitter crab” disease (Stentiford et al. 2001). However, even for this highly lethal pathogen of decapods, many of them with great commercial value (Stentiford & Shields 2005), the lifecycle and transmission strategies remain concealed (Li et al. 2021). It has been hypothesized that amphipods may play a major role in their life cycle as reservoirs (Small et al. 2006, Hamilton et al. 2009), and independent histological and molecular evidences have been observed, but are not conclusive (Johnson 1986, Pagenkopp-Lohan et al. 2012, Stentiford et al. 2012). In fact, it is possible that in many occasions

PCR positive samples and histological examinations correspond to genus *Syndinium*, sister to *Hematodinium* sp. (**Chapter 1**). Despite *Syndinium* spp. being long known parasites of copepods and ciliates (Chatton 1910, Soyer 1974, Coats 1999, Scovgaard et al. 2005), there is a single instance of this genus infecting amphipods (Manier et al. 1971). The authors described the pathogen *Syndinium gammari* from explants of *Gammarus locusta* in a Mediterranean lagoon. While no records of *S. gammari* exists since 1971 and its DNA has never been sequenced, it appears reasonable to think that the syndinian parasite infecting the *Gammarus* sp. population inabiting the Camel estuary (Cornwall) is indeed *S. gammari*, or a related novel *Syndinium* species. The histopathological, ultrastructural, and molecular analysis conducted by this study shows that the amphipod-infecting syndinian branches with the overlooked copepod-infecting *Syndinium* spp. These form a clade with several uncultured sequences, likely belonging to amphipod hosts as well. Thus, part of the enormous hidden diversity within clade Syndiniales may be concealed within amphipod hosts, highlighting the need for cautious identification of *Hematodinium*-like species. It should also be considered the potential of these overlooked pathogens to influence amphipod population dynamics.

Two different lineages of microcells belonging to late Supergroup Opisthokonta are responsible for recurrent infections in amphipod populations inhabiting the south coasts of the British Islands (**Chapter 1**). Numerous, extraordinarily diverse and intricately connected in the ecosystem, **Microsporidia** are regarded as emergent pathogens in the wild and the global food chains (Stentiford et al. 2019). However, they are often overseen by environmental DNA studies (Introduction – Section 4). From the 50 crustacean-infecting microsporidian genera, 13 are known to parasitize amphipods, totalling 30 described species, although amphipod-associated microsporidian diversity is likely five times greater (Bojko & Ovcharenko 2019). In spite of this, none of the microsporidian infections detected in coastal or estuarine amphipod populations in the south of the British Isles by this study are novel or cryptic species. Quite the contrary, amphipod-infecting *Dictyocoela* spp. are common throughout the British Isles and North-European coastal waters (Bacela-Spychalska et al. 2018). Parasitic in *Orchestia* sp., D. cavimanum appears to show inclination for talitrid amphipods (Wilkinson et al. 2011), while *D. duebenum* parasitizes predominantly gammarids. Species level identification (Chapter 1) for genus *Dictyocoela* is determinant, as vertically transmitted haplotypes tend to feminize infected hosts, while horizontally transmitted lines exert significant mortality in affected populations (Ironside & Alexander 2015, Pickup & Ironside 2018, Guler et al. 2018).

Hardly any endo-parasitic fungal-like infections have been documented in amphipods apart from microsporidians. So far, only *Candida gelida* and *Cryptoccous gammari* have been

identified (Segerstråle 1937, García et al. 2000, Bojko & Ovcharenko 2019). Similarly, only three ichthyosporean species are known to be associated to amphipods among holozoan protists (Glockling et al. 2013). Moreover, these ichthyosporean organisms are predominantly endosymbiotic and appear almost exclusively connected to the gut. Therefore, the interest arisen when an opisthokont-looking parasite was observed infecting the haemolymph, connective tissues, tegument, and gonads in amphipod genera *Echinogammarus* and *Orchestia*. The symbiont, turned out to be a novel species, *Txikispora philomaios,* which stands as the first confirmed parasite in Class Filasterea Cavalier-Smith 2008, and type species for novel genus *Txikispora* and Family **Txikisporidae** (Urrutia et al. 2021). Until the recent inclusion of *Pigoraptor* spp. by Hehenberger et al. (2017), Class Filasterea comprised only two genera: *Capsaspora* and *Ministeria* comprising three described species. The ecology of this clade remains essentially undetermined except for an apparent inclination for the low oxygen fraction from the water column in coastal waters of the Indian, Atlantic, and Pacific Oceans. However, this study further supports the inclusion of MAOP-1, an abundant clade of uncultured organisms into the clade (del Campo & Trillo 2013, Hehenberger et al. 2017).

The unearthing of *T. philomaios* illustrates the importance of combining histopathological analyses with molecular screenings to unveil the parasitic community associated to less habitually studied hosts, as it is the case of amphipods. Otherwise, highly divergent sequences are not confidently assigned to any clade and may end up passing over by sequence-rich environmental metabarcoding samplings (Arroyo et al. 2018). The initial phylogenetic analysis of *T. Philomaios* based on the complete 18S rRNA failed to include the pathogen within Filasterea. A multigene (96 genes) phylogenomic analysis was necessary to demonstrate the position of the amphipod-infecting holozoan within the class. This phylogenetic position was analogous to the one obtained when uncultured sequences were included in the 18S-based phylogenetic analysis. The outcome suggests that the use of environmental sequences in 18S phylogenetic studies provides additional phylogenetic information that may assist in resolving evolutionary relationships among holozoans, as it has been previously demonstrated for other eukaryotic groups (Berney et al. 2004, Cavalier-Smith 2004, Hartikainen et al. 2016, Bass et al. 2018).

The adjunction of *T. philomaios* to Filasterea, a clade constituted by free-living flagellated bacteriovores (except for the facultative endosymbiont *C. owczarzaki*), leaves the prospect of novel parasites being discovered within this group as a realistic working hypothesis. Further work will need to determine the extent and significance of cryptic filastereans as protists parasites. So far another 13 novel phylospecies have been phylogenetically placed into the group

in addition to MAOP-1 (Urrutia et al. 2021), most of them from a single study in Denmark, hinting a possible bias in the sequencing or the detection of filastereans in environmental samplings and their occurrence in other geographical locations. Rare and infrequent, there is no data on the temporal variability of this Class; this study constitutes the first one to reveal a temporal pattern in the abundance of a filasterean species. In fact, the strong but quickly vanishing prevalence peak observed for *T. philomaios* during May, exposes seasonality as an until now unaccounted bias for the scarcity of filastereans in environmental samplings (del Campo et al. 2015, Hehenberger et al. 2017, Mylnikov et al. 2019). Additionally, our failed efforts to amplify the 18S of *T. philomaios* from filtered water collected in Newton´s Cove during the peak of amphipod infection, reinforces the hypothesis of a reduced detection capability of eDNA for certain endo- parasites/symbionts (Dumonteil et al. 2018).

So far, both metagenomic and histopathological approaches analysing the pathogen community associated to amphipods are rare (Dattagupta et al. 2009, Abdelrhman et al. 2017, Chatterjee & Fernandez-Leborans 2013, Bojko et al. 2017, Bojko et al. 2019), especially those contemplating variation in a temporal scale (Messick et al. 2004). The seasonal analysis of amphipod genera *Echinogammarus* sp., *Orchestia* sp. and *Gammarus* sp. in Newton´s Cove conducted by this study constitutes the first general histopathological-molecular screening for these three genera in marine ecosystems (Bojko et al. 2017). It also outlines the importance of seasonality as a variable to examine the occurrence of certain parasitic lineages in amphipods, and possibly in other invertebrates. Additionally, it shows how parasites alternate between different amphipod species, often causing quick and virulent infections that can easily remain unobserved if not monitored at least on a monthly basis.

Seasonal differences in the incidence of protists parasites are particularly evident in host *Echinogammarus* sp., which apart from being one of the most abundant organisms in Newton´s Cove and coastal locations all over Europe (Maranhao et al. 2001) forms densely packed assemblages in the upper intertidal zone. While essentially affected by the same parasitic lineages, infections in *Gammarus* sp. and *Orchestia* sp. have steadier progress throughout the year, possibly due to a distinct diet, more sparsely distributed populations, or differences in susceptibility.

Ciliates have been observed to be the most prevalent protist lineage associated to amphipods, a finding that harmonizes with eDNA studies, which count Ciliophora as one of the most abundant protist lineages in coastal ecosystems (Zhang et al. 2018, Boscaro et al. 2019). Their prevalence appears to be higher during early summer, when the bacterial and micro-

plankton concentration is higher in the western English Channel (Rodríguez et al. 2000). This finding supports, the predominant commensalistic behaviour of amphipod-associated ciliates observed in the histopathological screening of amphipods in Newton´s Cove. More detailed molecular-based identifications could assist in identifying temporal changes in exclusively parasitic lineages (Prokopowicz et al. 2010, Chantangsi et al. 2013).

The incidence of **gregarine** endoparasite *Heliospora* sp. in amphipod genera *Gammarus*, and *Orchestia* remains rather steady throughout the year as well, with values ranging between 20% and 40%. In contrast, infection rates appear to fluctuate considerably in *Echinogammarus* sp. hosts. This prevalence values are similar to previous studies on amphipods (Grunberg & Sukhdeo 2017, Wróblewski et al. 2020), which identified host-size as the main driver; with adults being more frequently parasitized (Prokopowicz et al. 2010).

In contrast to **Ciliophora**, **Gregarinasin**a, and **Metazoa** (nematodes, copepods, digeneans), which in spite of some seasonal variability, appear associated to amphipods all year long, the incidence of **filasterean**, **haplosporidian**, **microsporidian**, **paramyxid, oomycetes**, and **syndinian** parasites differs substantially based on host species and season. In fact, there is a clear difference between the vigorous and short-lived microcell-caused diseases in *Echinogammarus* sp. versus the steadier development observed in genera *Gammarus* and *Orchestia*. Ascetosporean lineages Haplosporida and Paramyxida effectively exemplify this dichotomy. Both, *H. echinogammari* and *P. orchestiae*, infect between 5% and 15% of *Gammarus* sp. individuals virtually all year long, prevalence values slightly higher but consistent with other *Haplosporidium* spp. and *Paramarteilia* spp. infecting crustaceans, including amphipods (Feist et al. 2009, Stentiford et al. 2013, Winters & Faisal 2014, Davies et al. 2020). In contrast, *H. echinogammari* and *P. orchestiae* only infect *Echinogammarus* sp. in June and August/September respectively, when a significant part of the amphipod population becomes infected, especially in the case of the haplosporidian. While a much greater population density of this amphipod genus in Newton´s Cove an all over Europe (Martins et al. 2002) could explain these greater prevalence, identifying which factors determine the marked seasonality of ascetosporean infections in *Echinogammarus* is far more complex.

Factors influencing temporal variability of ascetosporean infections have only been investigated for commercially significant bivalves (Haskin & Andrews 1988, Robledo & Figueras 1995, Burreson& Ford 2004, Albuixech-Martí et al. 2020). Findings indicate that temporal variation of abiotic factors such as temperature or salinity alone do not explain disease outbreaks (Burreson & Ford 2004, Laing et al. 2014). Furthermore, studies usually discuss the

need of intermediate hosts or vectors to explain the completion of the parasite's life cycle and its seasonal variation (Arzul et al. 2014). Although, conclusive data are still needed, we have histological evidence of ciliates as vectors of haplosporidian cells, and PCR-based molecular proves of harpacticoid copepods either infected or transporting *Haplosporidium orchestiae*. Moreover, both ciliates and harpacticoid copepods have been shown to be especially prevalent during early summer, coinciding with the haplosporidian-infection outbreak in *Echinogammarus* sp. While further work is needed to confirm ciliates and/or copepods as possible vectors or intermediate hosts, high infection rates suggest that amphipods are not opportunistic hosts, but important reservoirs of ascetosporean parasites, with *Gammarus* sp. working as a **long-term pool** and *Echinogammarus* as a **seasonal amplifier**.

Regarding the temporal variability of opisthokonts, *Txikispora philomaios* represents the first confirmed parasite in Class Filasterea, rendering difficult comparison with related parasitic species (Urrutia et al. 2021). The small amoeba has been observed infecting genera *Echinogammarus* and *Orchestia*, but not *Gammarus*. Infection in Newton's Cove occurs almost exclusively during late April and May, being significantly higher (40-65%) in *Echinogammarus* sp. than in *Orchestia* sp. (~10%), possibly due to higher population density in the gammarid (Martins et al. 2002), variable feeding strategies, or differential susceptibility. Feeding strategies in *Echinogammarus* sp. include scavenging, predation, and cannibalism (Maranhão & Marques 2003, Dick et al. 2005, Alexander et al. 2012) bolstering a possible horizontal transmission by ingestion of infected prey or corpses. In contrast, *Orchestia* spp. are detritivorous/herbivorous, feeding predominately on algae (Moore & Francis 1985, Hines & Denno 2007), which combined to lower population densities might explain diminished *T. philomaios* prevalence.

The network analysis of co-occurrence between parasites conducted in *Echinogammarus* sp. hosts has shown that individuals parasitized by *T. philomaios* often present *Haplosporidium* sp. infections, as well. A co-infection occurrence that is greater than expected by chance (Pearson's Chy-squared test for independence, $p < 0.001$). Although the actual reasons remain unknown, in other parasitic protists including microsporidians, myxozoans, or coccidians, co-infection might be result of hyper-parasitism (Gómez-Couso et al. 2007, Stentiford et al. 2017, Sokolova & Overstreet 2020), a compromised immune system (Supali et al. 2010), or sharing a common vector/intermediate hosts (Poulin et al. 2003). So far, there is no microscopic evidence of hyper-parasitism, and potential intermediate hosts/vectors are yet to be confirmed for both parasites. Future work including transcriptomic analyses might reveal a down-regulation of genes involved in the normal functioning of the immune system in infected individuals.

The incidence of *Dictyocoela*-infections appears to be especially prevalent in *Echinogammarus* sp. during late summer (August/September), coinciding with infection by paramyxid microcells. In fact, this study indicates that both parasites tend to co-occur in the same host organism more frequently than expected by chance (Pearson's Chy-squared test for independence, $p < 0.001$) a widely documented association (Comps et al. 1980, Villalba et al. 1997, Short et al. 2012). Furthermore, *Dictyocoela* sp. and *Paramarteilia* sp. parasites have been linked to feminization of infected amphipods, possibly as a way of increasing vertical transmission (Ironside & Alexander 2015). Although, more recently, the feminizing effect has been linked exclusively to the paramyxid (Pickup & Ironside 2018, Guler et al. 2018). Although, the peak of microsporidian and paramyxid co-infection does not seem to be associated to a significant increase in the ratio of intersex individuals, the decrease in the number of larvae-bearing females observed by this study is unquestionable. Since a larvae-hatching collapse by female castration would be rather illogic in these vertically transmitted parasites (Dunn et al. 2001, Ward et al. 2016), and sex ratio distortion causing incomplete feminization of males (Cormier et al. 2021) has not been observed, it is possible that the microsporidian is changing transmission strategy in *Echinogammarus* sp. hosts. Equivalent changes in transmission strategy from vertical to horizontal have already been described among microsporidians (Haag et al. 2020) and could represent a way to infect other amphipod hosts such as *Gammarus* sp., a likely reservoir for various *Dictyocoela* spp. in Newton's Cove. Besides, a deadlier stage of horizontal transmission in *D. duebenum*-infected *Echinogammarus* sp. (Guler et al. 2018) could explain the reduction in the number of amphipod larvae and consequently the end of vertically transmitted *P. orchestiae* infections observed towards the end of September in the population. However, future work will be needed to substantiate this new hypothesis.

The incidence of **oomycete** and fungal infections in amphipods from Newton's Cove is low (< 5%), suggesting that they represent opportunistic hosts rather than reservoirs. However, it is possible that oomycete and fungal parasites are more prevalent in other amphipod species present in Newton's Cove or the surrounding area. So far, the taxonomy of these parasites remains unknown, but the occurrence of oomycete and fungal infections in crabs and lobsters in this area of the English Channel (Stentiford et al. 2003, James et al. 2017, Holt et al. 2018, Davies et al. 2020), advice for a detailed phylogenetic study and more screenings. This is in case that the amphipods represent intermediate hosts or vectors for these lineages, as already hypothesized by other authors (Sarowar et al. 2013, Svoboda et al. 2014, Bojko & Ovcharencko 2019).

Infections caused by micro-eukaryotes in the amphipod community from Newton´s Cove have been shown to be governed by seasonality and to progress differently in each host. A natural ending for this thesis was to evaluate whether the temporal variability observed for protist infections in the amphipod community inhabiting Newton´s Cove were extrapolatable to other locations in the south of the British Isles.

In the case of Ciliophora, the main difference between amphipods from Newton's Cove and populations inhabiting Tamar, Dart, and Camel estuaries, is that in the later, ciliates are more abundant in Spring (April) than in Summer (June). Predominantly ectocommensalistic (although the cuticle is often penetrated), these higher prevalence have been linked to increased levels of phytoplankton and bacteria in the sediment (Pitsch et al. 2019), which in estuaries in the south of England, are more exuberant and occur earlier than in exposed coastal habitats (Kocum et al. 2002, Cloern et al. 2014). Additionally, almost all amphipod-associated ciliate genera identified in amphipods (by metagenomic analysis) belong to Classes Phylopharingea and Oligohymenophorea, which are considerably more abundant in estuarine waters than in euhaline coastal habitats (Urrutxurtu et al. 2003, Sun et al. 2017).

In turn, the prevalence of gregarines infecting estuarine amphipod populations is substantially higher than the one observed in amphipods inhabiting the coast. So far, only a handful of works have analyzed host-associated gregarine prevalence on a spatial level, for the most part in commercially important invertebrate clades including crabs (Messick et al. 1998), cockles (Carballal et al. 2001), or oysters (Winstead et al. 2004). However, spatial changes in the prevalence of amphipod-infecting gregarines remained until now undocumented. One of the better studied gregarine genera is *Nematopsis*, which has been shown to be negatively correlated to higher salinities, in *Mytilus* sp. mussels (Kovačić & Pustijanac 2017), and *Litopenaeus* sp. shrimps (Jimenez et al. 2002). A comparable behaviour for amphipod-infecting gregarines would back up the higher prevalence observed in estuarine ecosystems. Besides, the positive correlation between size and gregarine prevalence suggested (Prokopowicz et al. 2010, Grunberg & Sukhdeo 2017) would be consistent with this hypothesis, as all three amphipods studied tend to grow slower but bigger in estuarine waters (Maranhão & Marques 2003).

Ascetoporean lineages **Microsporidia** and **Paramyxida**, cause infections in all amphipod genera and locations investigated. Their occurrence and prevalence explain, together with gregarines, most of the variability observed (in a PCA) between estuaries and Newton's cove. Both parasites, which are especially prevalent during late summer, appear to be more abundant in estuaries than in coastal habitats. Furthermore, fundamentally identical microsporidian and

paramyxid infection prevalence in *Echinogammarus* sp. regardless of season and location substantiate the co-occurrence of both parasites above discussed. Amply investigated (Terry et al. 2004, Wilkinson et al. 2011, Ironside & Pickup 2015) these co-infection remains fundamentally uncharacterized in a temporal and geographical basis (Guler et al. 2018). Co-infection, which in Newton´s Cove *Echinogammarus* sp. population is restricted to late summer, is clearly bimodal in estuarine populations. Differences in the population dynamics of the amphipod between estuaries and coastal habitats, or in the occurrence of intermediate hosts, might explain the existence of this second peak of co-infection during winter in estuaries. Independently of the reasons driving these differences in prevalence, the results show that estuarine *Echinogammarus* sp. populations represent a reservoir of *Paramarteilia orchestiae* and *Dictyocoela* sp. all year long, contrasting with coastal populations.

Although statistical analyses have shown certain level of co-infection by *Dictyocoela* sp., and *P.orchestiae* in the other two amphipod genera investigated (*Gammarus* and *Orchestia*) both parasites are known to cause independent infections in these and other amphipod and crustacean species (Bacela-Spychalska et al. 2018). The handful of studies discussing spatial and temporal variability of *Dictyocoela* spp. suggested that lower temperatures might inhibit the parasite's replication (Dunn et al. 2006. Ryan & Kohler 2010, Quiles et al. 2020). However, although this finding would be consistent with maximum infection prevalence observed in coastal amphipod populations during late summer, it would fail to explain the second infection peak observed during winter in estuaries.

The spatiotemporal variability of *Paramarteilia*-caused infections in crustaceans is limited to few observations in amphipods and crabs (Feist et al. 2009, Short et al. 2012, Ward et al. 2016). The incidence of *P. canceri* infecting crabs in southwest England has been shown to be slightly higher during winter (Feist et al. 2009), and Ward et al. (2016) observed *P. orchestiae* to be more prevalent in the Gann estuary than in a coastal locations. Their results are consistent with findings by the present study, showing a preference of *P. orchestiae* for estuarine amphipod populations or estuarine conditions and a two-peak infection. This putative inclination for estuarine habitats is possibly shared by related genus *Marteilia*, observed to be more prevalent in mussels collected in estuaries (Tamar), than in coastal locations nearby (Bignell et al. 2011). On the one hand, it is evident that a better comprehension of *Paramarteilia* sp. cycle will be necessary to grasp the drivers influencing the appearance and development of the disease in amphipods, crabs, and maybe other invertebrate hosts. On the other hand, our results advise against analyzing these two infections (Microsporidia and Paramyxida) separately in populations

where co-infection has been documented or remains undetermined, as the mechanisms driving co-occurrence are not fully comprehended yet (Guler et al. 2018).

In line with ciliates and gregarines, haplosporidian infections appear to surge earlier in amphipods inhabiting estuaries than in Newton's Cove. For instance, *Haplosporidium echinogammari* which is almost exclusively observed during June in the *Echinogammarus* sp. population from Newton's Cove, occurs in estuarine populations throughout the year, with peaks of infection occurring earlier during spring. In turn, *Haplosporidium orchestiae,* which is not present in coastal waters (Newton's Cove), infects *Orchestia* sp. populations in all three estuaries analysed, being more prevalent during the first half of the year. Until the description of *H. echinogammari* and *H. orchestiae* (Urrutia et al. 2019), the only amphipod-infecting haplosporidian species described was *Haplosporidium diporeiae*, which causes disease in the freshwater genus *Diporeia* from the Great Lakes (USA). However, no spatial trends have been observed in the distribution of this parasite in its type-location (Winters & Faisal 2014). Similarly, the other three *Haplosporidium* spp. causing infections in crustaceans, *H. littoralis*, *H. carcini*, and *H. cranc*, are only known from their type locations in the British Isles (Stentiford et al. 2013, Davies et al. 2020). In contrast, the spatiotemporal distribution of more extensively studied bivalve-infecting haplosporidians, such as Haplosporidium nelsoni is known to be regulated by salinity and to lesser extent by (Ford & Haskin 1988, Carnegie & Burreson2011). The parasite, in consonance with *H. echinogammari* has been noticed to be especially prevalent in estuaries during early summer, when water temperature is increasing (Ford 1985, Barber et al. 1997). However, the role of ciliates and/or copepods as potential vectors/ intermediate hosts of *Haplosporidium* spp. also gets substantiated by this premature peak of infections in estuaries, as it co-occurs with observed ciliate and zooplankton heights (Kocum et al. 2002, Cloern et al. 2014).

The presence of the novel amphipod-infecting parasite *T. philomaios* is not restricted to Newton's Cove. In fact, infections caused by the **filasterean**, which in Weymouth occur almost exclusively during May, have been microscopically detected throughout the year in Tamar and Dart estuaries, although its predominant prevalence during Spring is evident. The quarterly analysis conducted in estuaries does not allow addressing some important questions: The existence in estuaries of equivalent annual outbreaks to those observed in Newton's Cove or the reasons for infection prevalence to be higher in estuarine populations of *Orchestia* sp. Than those of *Echinogammarus* sp. when in Newton's Cove *Orchestia* sp. was uninfected. So far, it has been shown that *T. philomaios* is able to infect at least two different amphipod genera, indicating certain range of hosts specificity, that could expand notably if molecularly deteceted

infection in Procerodes sp. is confirmed by histology. If uninfected, the turbellarian could still represent a mechanical vector facilitating the dispersal of viable T. philomaios cysts.

In general, the community of amphipod-infecting protists parasites observed in Newton's Cove appears to be representative of the main micro-eukaryotic infections affecting amphipods inhabiting marine and estuarine ecosystems in the southwest coast of England. The spatiotemporal changes in the prevalence of ciliate, gregarine, filasterean, haplosporidian, microsporidian, and paramyxid protists highlight the rapid shifts in the occurrence of some protist parasites, which temporal distribution is being increasingly documented by molecular methods, but seldom associated to infection (Berdjeb et al. 2018, Sassenhagen et al. 2020). The rapid generation time of some protists, in some cases spanning less than a day (Ohtsuka et al. 2016), promotes rapid swings in their prevalence. These ephemeral populations, which might last between one and three weeks (Vigil et al. 2009) constitute an evident bias for all but recurrent temporal analyses (Simon et al. 2015).

Considering all locations and seasons, populations of *Echinogammarus* sp. are slightly more parasitized than those of *Orchestia* sp., and considerably more parasitized than those of *Gammarus* sp., a difference that is statistically significant (p-value < 0.05, One-way Anova; $p < 0.05$, Tukey HSD test) when micro and macro-eukaryotic parasites are considered. The reduced number of general screenings for parasites in amphipods (Winters et al. 2014, Bojko et al. 2017), anticipate the lack of comparable studies analysing the parasitic load by host in this clade, a paucity that is extensive to other crustacean hosts as well (Stentiford & Feist 2005, Wolinska et al. 2011). Although the myriad of drivers (size, host ratio, diet, immune system, population density) can render interpretation a complex enterprise (Vestbo et al. 2019), differential parasitisation-levels between individual hosts, populations, or lineages have been shown to rule invasions, population dynamics, and ecology (Poulin & Morand 2000). The differential distribution of parasitic spores and/or intermediate hosts between upper and lower intertidal zones could easily explain our observations (Hall et al. 2005, de Montaudouin et al. 2012); not to mention the above discussed inter-specific variability in diet, sex-ratio, immunity, age, or reproductive cycle among many other variables. However, the interest and impact of parasitic load in the spatial distribution of a host population at large-scale is well illustrated by invasive species. There is mounting evidence demonstrating the weight of the pathobiome in the success, or failure, of species expanding their range or invading different continents (Gendron et al. 2012, Young et al. 2017, Lagrue 2017), amphipods included (MacNeil et al. 2003, Prenter et al. 2004, Kestrup et al. 2011).

There is no significant difference in the total pathogen load between seasons, when estuarine and coastal populations of the three amphipod genera investigated are grouped together (p-value > 0.05, One-way Anova). Which does not mean that there are no differences in the occurrence and prevalence of certain amphipod-infecting protists clades between seasons, as already discussed. In fact, this study shows how general or specific screenings for protist parasites could be especially biased during spring and summer, when communities change more and quicker in amphipods, and in the environment (Berdjeb et al. 2018), outlining the need for additional sampling effort during this time of the year. The data also show an apparent "stability" in the seasonal parasitic load sustained by amphipods in this area of the UK. While clearly insufficient to draw equivalent hypotheses and explanations, these results recall those suggested by archetypal modelling studies (Anderson & May 1981), in which stabilized host populations might be in equilibrium with their parasitic burden given certain conditions, including parasites exerting mortality.

Predominantly driven by higher incidences of microsporidian, paramyxid, and gregarine microcell parasites and nematodes, amphipods from estuarine waters are significantly more parasitized than those from coastal waters (p-value < 0.05, Kruskal-Wallis test; p < 0.05, pairwise Wilcox test). In fact, differences (Bray-Curtis dissimilarity) in the occurrence and prevalence of protist parasites allow to distinguish (with certain overlap) between estuarine and coastal (Newton's Cove) host populations. Comparable results have been shown to consent a fine-scale spatiotemporal assessment of distribution and migration routes in fish populations (Levy et al. 2019, Lennox et al. 2020). Furthermore, equivalent histopathological analyses of other significant macro-benthic species, could procure a vision of the general health status and host-stress in different locations (Stentiford et al. 2003, Stentiford & Feist 2005). Insights that would certainly have an impact environmental assessment studies (Lafferty 1997, Marcogliese & Pietrock 2011), and influence decision making in fields ranging from aquaculture, and feeding industry to harbour/inner waters management (dredging, river transfers, ballast waters).

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CONCLUSIONS AND THESIS

CONCLUSIONS

1. The invertebrate community in the selected type location in the south of the British Isles (Newton´s Cove) was dominated by crustaceans (amphipods, copepods, and isopods), annelids (polychaetes), platyhelminths (turbellarians), molluscs (gastropods), nemerteans, and nematodes. This macrobenthic structure is illustrative of the prevailing ecological assemblages found in temperate rocky coasts in general and in the British Isles in particular. Species-richness and alpha diversity develop towards the lower-levels of the intertidal zone, where the number of rare species is highest. Most abundant species thrive in the upper belt of the intertidal zone, where stressful abiotic conditions halt greater evenness.

2. Ecological dominance in macrobenthic assemblages in the upper intertidal zone was exerted by four main invertebrate genera: *Echinogammarus* (Amphipoda: Crustacea), *Capitella* (Polychaeta: Annelida), *Procerodes* (Turbellaria: Platyhelminthes) and ameirid harpacticoids (Copepoda: Crustacea). On top of their significant abundance (> 1000 individuals m^{-2}), these four lineages play a pivotal role in the food-web of temperate coastal ecosystems, thus representing a clear link between different trophic levels and allowing comparison at large spatiotemporal scales.

3. *Echinogammarus* sp. is an ecologically relevant but overlooked reservoir host for a sizeable number of micro-eukaryote parasites, as evidenced by histopathological examination of dominant macrobenthic taxa (*Echinogammarus* sp., *Capitella* sp., *Procerodes* sp., and harpacticoid copepods) along various seasons.

4. A great diversity of amphipod-infecting protist lineages, including Ciliophora, Apicomplexa, Microsporidia, Endomyxa, Syndiniales, Oomycetes, and Filasterea was revealed after combined histopathological, ultrastructural, and molecular analyses of the parasitic community associated to *Echinogammarus* sp., and other common amphipod genera (*Gammarus* and *Orchestia*).

5. Contrary to ciliates, gregarines, and metazoan parasites (nematodes, copepods, and digeneans), which in spite of some seasonal variability appeared associated to amphipods all year long; the incidence of filasterean, endomyxid, microsporidian, oomycete, and syndinian parasites differed substantially based on host species and season. *Echinogammarus* sp. populations were characterized for hosting high-incidence, abrupt, and virulent protist infections that followed a rather predictable pattern; filastereans in May, haplosporidians in June, and paramyxid-microsporidian co-infections in late summer. Infections by these same protist lineages in *Gammarus* sp and *Orchestia* sp. populations, which form significantly sparser assemblages, had a steadier progress and prevalence.

6. The spatiotemporal comparison of these infection dynamics in neighbouring locations (South of the British Isles)showed that amphipod populations inhabiting estuaries are significantly more parasitized than their coastal counterparts; mainly due to a seasonally bimodal distribution in the development of infections. *Echinogammarus* sp. was slightly more parasitized than *Orchestia* sp.; and both significantly more parasitized than *Gammarus*

sp. These inter-taxa differences in the parasitic load remained irrespective of the season and location

7. An amphipod-infecting syndinian was sequenced for the first time, providing novel evidence of amphipods as hosts for a key parasitic clade comprising great hidden diversity. Syndinian-caused parasitisation, infrequent in the amphipod populations studied, was exclusively observed in *Gammarus* sp. The infection consisted of parasitic trophonts that grow and reproduce in the host haemolymph until they cause congestion of haemal sinuses.

8. Amphipod-infecting haplosporidians (Ascetosporea: Endomyxa: Rhizaria: TSAR Supergroup) have been recorded for first-time in the temperate ecosystems (British Isles). Actually, the phylogenetic analyses indicate that such infections are caused by at least two novel and highly-divergent species: *Haplosporidium echinogammari* n. sp. and *Haplosporidium orchestiae* n. sp., parasitizing *Echinogammarus* sp. and *Orchestia* sp. populations respectively.

9. Twenty-five different and novel haplosporidian genotypes associated to crustacean hosts have been sequenced, with virtually all of them clustering within three crustacean-rich *Haplosporidium* sp. lineages. Phylogenetic and histopathological analyses of a variety of amphipods, crabs, crayfish, and isopods collected in marine and freshwater ecosystems from Europe and North America, indicate that part of the hidden diversity within the clade of haplosporidians, reckoned mollusc-infecting parasites, is hosted by crustaceans as well.

10. Novel amphipod-infecting species *Txikispora philomaios* (Txikisporidae; Filasterea; Opisthokonta), constitutes the first confirmed parasite within Class Filasterea, which together with choanoflagellates represents the closest protistan relatives of metazoans. The parasite, smaller than its filasterean counterparts and most holozoans (2.3 -2.6 µm), causes infection (often intracellular) in the haemolymph, connective tissue, integument, gonads, hepatopancreas, and nervou tissue of genera *Echinogammarus* and *Orchestia*. Ultrastructural analyses have shown cellular division inside host tissues, the development of cysts, and a likely flagellar structure (substantiated by the presence of genes constituting the flagellar toolkit).

11. The position of *Txikispora philomaios* as the earliest diverging-branch within Filasterea, has been resolved by phylogenetic and phylogenomic analyses; showing that 18S phylogenetic studies including uncharacterized environmental sequences can provide additional phylogenetic information that may assist on determining evolutionary relationships. Following this approach, at least thirteen previously unknown lineages from environmental samples have been included within Filasterea, which until now was constituted by 5 species. The parasitic lifestyle of *T. philomaios* makes a realistic working hypothesis, the potential as parasites of some of these hidden filasterean lineages.

THESIS

Amongst common invertebrate species inhabiting the intertidal zone in temperate coastal ecosystems, amphipods represent an ecologically relevant but overlooked reservoir for a significant number of micro-eukaryotic parasites of concern for marine environment and resources, including alveolate, rhizarian, and opisthokont lineages

The detection, identification, and characterization (histopathological, ultrastructural, and phylogenetic) of these parasites and their association to amphipods has permitted to contextualize existing and hidden diversity, whilst revealing novel parasite species and lineages too. In parallel, screening of co-occurring dominant invertebrate hosts (copepods, polychaetes, and platyhelminthes) by microscopic and molecular approaches reveals clues for understanding the transmission routes, seasonal patterns of infection, and natural variability of these parasites in temperate coastal ecosystems.


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