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Anna-Lotta Hiillos

Disentangling Symbiont-host Interactions in a Group of Understudied, Putative Parasites

The Marine Apicomplexa

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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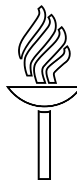

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ABSTRACT

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Symbiotic interactions (antagonistic, synergistic, or neutral) have been of fundamental importance in shaping evolution of their hosts as well as other symbionts infecting the same host. Understanding the diversity, drivers and outcomes of these interactions is important in resolving species capability to adapt in changing environments. Apicomplexans are known to infect a wide variety of marine invertebrates, but their diversity and how they affect their hosts' fitness is unclear. Are they parasites or mutualists? In this thesis, I aimed to disentangle these interactions between two marine apicomplexans, *Rhytidocystis* sp. and *Selenidium pygospionis*, infecting a marine polychaete. Using molecular methods, I surveyed natural host populations for infection dynamics, coinfection dynamics and how they are affected by different host characteristic. In addition, I described apicomplexan richness in relation to their host species richness on a large spatial scale, the Baltic Sea salinity gradient. I found that infection patters vary spatially and temporally and are affected by host size, but independent of host population density and genetic diversity. The spatial differences are likely due to differences in local environmental factors affecting symbiont transmission. I also found signs of synergistic (beneficial) interactions between the two symbionts while coinfecting the same host. In addition, I found indications that the richness of host communities might inhibit infection success (dilution effect). Overall, this thesis describes the infection patterns of the understudied apicomplexans, gives an indication of factors affecting the interactions between them and their host, as well as how other factors might affect their infection success in ecologically important benthic animals. However, this thesis also emphasizes that resolving the nature of symbiotic interactions is difficult with only direct observations from nature and controlled experimental approaches are required to gain a deeper understanding of these relationships.

Keywords: Apicomplexa; coinfections; dilution effect; diversity; infection dynamics; symbiont-host interactions.

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TIIVISTELMÄ

Hiillos, Anna-Lotta
Symbionttien ja isäntien väliset vuorovaikutukset heikosti tunnetussa, oletettavasti loisivassa lajiryhmässä: merissä esiintyvät Apicomplexa -suvun symbiontit
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Vuorovaikutukset symbionttisten eliöiden ja niiden isäntien välillä (antagonistiset, synergistiset tai neutraalit) ovat olleet perustavanlaatuisessa asemassa kautta eliöiden evolutiivisen historian. Näiden vuorovaikutusten moninaisuuden ja niitä ylläpitävien tekijöiden tunteminen on tärkeää, jotta lajien sopeutumiskykyä muuttuviin elinolosuhteisiin voidaan ymmärtää. Apicomplexa sukuun kuuluvat eliöt ovat yleisiä merissä elävien selkärangattomien symbiontteja, mutta niiden vaikutusta isäntänsä kelpoisuuteen ei tunneta. Tässä väitöskirjassa tutkin kahden Apicomplexa -suvun symbiontin vuorovaikutusta niiden isäntänä toimivan hiekkaputkimadon kanssa, sekä symbionttien välisiä vuorovaikutuksia eri DNA-menetelmien avulla. Tutkin symbionttien infektiotehokkuutta, sen määrää, sekä siihen vaikuttavia isäntien ominaisuuksia luonnollisissa isäntäpopulaatioissa. Lisäksi tutkin Apicomplexa -suvun symbionttien monimuotoisuutta suhteessa niiden isäntälajien monimuotoisuuteen Itämeren pohjaeläinyhteisöissä. Tulokseni osoittivat, että symbionttien infektiotehokkuus vaihtelee paikallisesti ja vuodenajan mukaan. Isännän koko vaikuttaa infektion todennäköisyyteen, mutta populaatiotiheydellä tai geneettisellä monimuotisuudella ei ollut vaikutusta. Nämä erot saattavat johtua paikallista ympäristötekijöistä, jotka vaikuttavat symbionttien transmissioon. Korkea lajien monimuotisuus isäntäpopulaation elinympäristössä puolestaan vaikutti alentavan symbionttien infektiotehokkuutta, mahdollisen diluutioefektin vaikutuksesta. Lisäksi tulokseni viittaavat hyödylliseen vuorovaikukseen symbionttien välillä niiden infektoidessa samaa isäntäyksilöä (koinfektio). Väitöskirjani antaa uutta tietoa tekijöistä, jotka vaikuttavat näiden heikosti tunnettujen symbionttien esiintymiseen ja infektiotehokkuuteen luonnossa. Toisaalta tutkimukseni osoittavat, että vuorovaikutusten tutkiminen luonnossa ilman kokeellisia menetelmiä on haastavaa.

Avainsanat: Apicomplexa; diluutioefekti; infektiodynamiikka; koinfektio; monimuotoisuus; symbioottiset vuorovaikutukset.

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ORIGINAL PAPERS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I–III.

I Anna-Lotta Hiillos, Anne Thonig and K. Emily Knott. 2021. Droplet digital PCR as a tool for investigating dynamics of cryptic symbionts. *Ecology and Evolution* 11: 17381–17396.

II Anna-Lotta Hiillos, Irin Rony, Sonja Rueckert and K. Emily Knott. 2022. Coinfection patterns of two marine apicomplexans are not associated with genetic diversity of their polychaete host. *Journal of Eukaryotic Microbiology,* 00, e12932. doi: 10.1111/jeu.12932

III Anna-Lotta Hiillos, H. Cecilie Petersen, Benni W. Hansen, Gary Banta, Sonja Rueckert and K. Emily Knott. Benthic community diversity indicates diversity of marine Apicomplexa in coastal sites of the Baltic Sea, but dilution effects drive infection patterns. Manuscript.

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Finalizing	all authors	all authors	all authors

ALH = Anna-Lotta Hiillos, KEK = K. Emily Knott, AT = Anne Thonig, SR = Sonja Rueckert, HCP = H. Cecilie Petersen, GB = Gary Banta, BWH = Benni W. Hansen, IR = Irin Rony

ABBREVIATIONS

bp	Base pair
cox1	Cytochrome *c* oxidase subunit I gene
CV%	Coefficient of variation percentage
ddPCR	Droplet digital PCR
DNA	Deoxyribonucleic acid
EtOH	Ethanol
HTS	High throughput sequencing
LoD	Limit of detection
NGS	Next generation sequencing
ng	Nanogram
PCR	Polymerase chain reaction
RFU	Relative fluorescence unit
μl	Microlitre
μM	Micro molar

1 INTRODUCTION

1.1 Symbiont – host interactions continuum

The term symbiosis was first defined by the botanist Anton de Bary in 1879 as the intimate "living together of two differently named organisms" (Sapp 2004). These interactions nowadays refer to a variety of associations between species ranging from parasitism, where one organism gains benefit at the expense of the other, to mutualism, where all parties benefit (Tipton *et al.* 2019). Interactions between symbiotic organisms and their hosts are recognized as significant drivers of ecological and evolutionary processes. In fact, the importance of symbiosis on the evolution of eukaryotic life is highlighted by the origin of eukaryotic cells by endosymbiosis of prokaryotic cells, which later evolved to specialized organelles, such as the mitochondria and chloroplasts (Sagan 1967, Roger *et al.* 2017). Symbiotic interactions can modify the physiology of the interacting partners (Dunbar *et al.* 2007), alter the behaviour of their hosts (Wood *et al.* 2007), affect the interactions and distribution of species (Flórez *et al.* 2015, Freeland 1983, Hatcher *et al.* 2006) and ultimately shape biodiversity on Earth (Chomicki *et al*. 2019, Hembry *et al*. 2016). However, in many cases the outcome of the symbiosis does not necessarily have any noticeable effect on either of the interacting parties. Symbiotic interactions often are also dynamic and changes in environmental conditions might trigger an otherwise neutral or beneficial symbiosis to parasitic (Overstreed and Lotz 2016) and vice versa, as many mutualistic interactions are thought to have evolved from parasitic origins (Weiblen and Treiber 2015, Sachs *et al*. 2014). For example, scleractinian corals are dependent upon symbiotic dinoflagellates for energy and cannot grow without them. Fuess *et al*. (2020) found that increasing nutrients in the environment led to an increase in symbiont density, which in turn lowered the host corals immunity to a disease. Another example comes from gregarines infecting the mustard leaf beetle: the symbionts had no effect

on their hosts fitness under normal nutritional state, but when the hosts were under stress from starvation, their reproduction and survival was lowered by the gregarine infection (Woltz *et al.* 2022). Regarding the vast diversity in symbiotic interaction outcomes and their continuum-like nature, throughout this thesis the term "symbiotic interactions" does not refer to any specific relationship (beneficial or antagonistic) between the interacting species.

1.1.1 Parasitism

Parasitism occurs when an organism lives at the expense of another. Parasitic symbionts cannot live without their host and usually cause some harm to their host's fitness (antagonistic interaction). Antagonistic interactions usually involve an arms race between the host and the parasite whereby the parasite evolves new means to counteract the host's defensive strategies (Anderson and May 1982, Sheldon and Verhulst 1996). As parasitism has been hypothesized to be the most common form of living on Earth (Poulin 1996, Windsor 1998), these interactions can be considered as one of the most important symbiont-host interactions in shaping biodiversity. Parasites can, for example, regulate their host's population dynamics (Anderson and May 1978, May and Anderson 1978), affect the outcome of interspecific competition (Price *et al*. 1986, Tompkins *et al*. 2003) and predator–prey interactions (Hatcher *et al*. 2006), and, consequently, can alter the whole community structure and functioning (Mouritsen and Poulin 2005, Real 1996).

Infection by a parasite is determined mainly by two factors. First, parasites need to encounter a host. Encounter can either occur vertically, where parasites are directly inherited from the previous generation of hosts, or horizontally, where infective stages of the parasites are acquired from the environment or from contact with an infected individual (Combes 2001). For example, the arthropod reproductive parasite, *Wolbachia*, is vertically transmitted from the reproductive tissues of infected mothers through the egg cytoplasm (Werren *et al*. 1995). An example of horizontally transmitted parasites are the coccidian protists causing coccidiosis in many mammals. These parasites transmit via an oral-fecal route by which the host accidentally ingests coccidian infective stages (oocysts) from the environment while grazing (Belli *et al*. 2006).

After the encounter, susceptibility to the infection determines if a host becomes infected of not (Combes 2001). Susceptibility of a host to a specific parasite can vary greatly between individuals (Hassell and Anderson 1984) as it depends on various factors that affect the hosts capability to resist the infection or to limit its pathogenicity. Such factors include for example the genetic composition of both host and the parasite, specific immunity (production of antibodies), host sex, age, and nutritional status (Cornet *et al*. 2014, Ganz and Ebert 2010, Izhar and Ben-Ami 2015, Zuk and McKean 1996).

1.2 The Apicomplexa

Apicomplexans are single-celled eukaryotes that are obligate endosymbionts of many vertebrate and invertebrate taxa (Morrison 2009). The phylum has been considered exclusively parasitic due to the vast number of highly pathogenic and medically and economically important species, such as the parasites causing malaria (*Plasmodium* spp.), toxoplasmosis (*Toxoplasma gondii*), babesiosis (*Babesia* spp.) and coccidiosis (*Eimeria* spp.) (Seeber and Steinfelder 2016). Currently, approximately 6,000 species in more than 300 genera have been named (Adl *et al.* 2019). However, their diversity is likely highly underestimated, since research on this group has been greatly biased towards the economically important host species. As research on apicomplexans infecting host organisms that are not of economic importance has only recently begun to grow interest, only an incomplete record exists. This is the case especially in marine environments in which the diversity is largely undescribed and is suspected to contain many cryptic species (Janouškovec *et al*. 2015, Xavier *et al*. 2018). It is thought that every animal species hosts at least one apicomplexan (Morrison 2009, Votýpka *et al*. 2017) and in many cases, a single host species has been found to be infected by multiple distinct apicomplexan species (e.g., Bunker *et al.* 2013, Grunberg and Sukhdeo 2017, Maia *et al*. 2014, Paskerova *et al.* 2018). Additionally, despite the assumption that all apicomplexans are parasitic, for some groups the effects they have on their hosts have not been studied and the nature of the interaction remains unclear. One such group is the gregarines (Rueckert *et al*. 2019).

1.2.1 The gregarines

Gregarines are a group of endosymbionts in the phylum Apicomplexa that infect many marine, freshwater, and terrestrial invertebrate species. Gregarines primarily infect the intestines, coeloms or reproductive vesicles of their hosts (Desportes and Schrével, 2013). Most gregarines complete their development within a single host individual (direct life cycle) and spread through the release of highly environmentally persistent oocysts containing the infective stages (sporozoites) among the feces of the host (Clopton 2002, Logan *et al.* 2012). Their transmission to a new host then occurs when another host ingests the oocysts, from which the sporozoites emerge and transmit to the appropriate tissue. Historically, this group has been divided into three categories based on habitat, host range and trophozoite (feeding stage) morphology: archigregarines, eugregarines and neogregarines (Desportes and Schrével 2013, Leander 2008). Archigregarines are characterized by a similar morphology throughout their development and exhibit dynamic coiling movements. These symbionts occur exclusively in marine environments infecting mostly annelids and are considered, together with *Cryptosporidium*, to be the earliest diverging lineages within the Apicomplexa (Desportes and Schrével 2013, Leander 2008, Leander *et al.* 2003, Schrével *et al.* 2016). Eugregarines infect invertebrates in marine,

freshwater and terrestrial habitats. These symbionts differ in both their morphology and behaviour between the developmental stages. Neogregarines are predominantly intracellular parasites and found only infecting terrestrial insects (Desportes and Schrével 2013).

Gregarines are thought to be highly host-specific (Clopton and Gold 1996, Perkins *et al*. 2000). Many studies have investigated the interactions between gregarines and their hosts from the perspective of parasite-host coevolution as well as for their potential as biological controls of pest insects (Alarcón *et al*. 2011, Detwiler and Janovy 2008, Lantova and Volf 2014, Tseng 2007). However, knowledge on how majority of gregarines affect their hosts' fitness is still limited and the interactions can range from mutualistic to parasitic depending on the system (Rueckert *et al.* 2019). This is the case especially for aquatic gregarines. Only a few studies based on observations on natural host populations have reported detrimental effects of gregarine infection on host fitness, but in most studies the effects are mild or seem negligible. For example, the gregarine *Lankesteria ascidiae* was found to cause high mortalities in ascidians (Mita *et al.* 2012), while the gregarine *Nepatopsis* sp. has been reported to cause mild or non-noticeable pathogenicity to its bivalve hosts (Kua *et al*. 2013, Uddin *et al.* 2010). In terrestrial gregarines, the effects on their host's fitness are better known due to possibility of experimental infection studies. These effects have been reported to be antagonistic, such as shortened lifespan and growth, higher mortality, and nutrient deficiency (Gigliolli *et al*. 2016, Schilder and Marden 2007), neutral (Klingenberg *et al.* 1997) or even beneficial, such as faster developmental time, better survival under stress from starvation (Alarcón *et al*. 2017, Arcila and Meunier 2020, Valigurová 2012). The fact that in many cases the gregarines do not seem to have any effects on their host's performance might lead to overlooking the infection (Klingenberg *et al*. 1997, Tsubaki and Hooper 2004).

1.2.2 The Marosporida

Apicomplexan species in the genus *Rhytidocystis* are coccidian-like symbionts that infect the midguts of marine polychaetes (Leander and Ramey 2006, Miroliubova *et al.* 2020, Rueckert and Leander 2009). Currently, only a small number of species in this genus has been described. This group was previously assigned to the coccidian family Agamococcidiorida along with a single species containing genus *Gemmocysis* (Levine 1979, Upton & Peters 1986). However, this placement has recently been challenged and based on multiprotein phylogenomics, a new order, Marosporida, has been established where rhytidocystids are placed together with other marine invertebrate infecting apicomplexan genera *Aggregata*, *Merocystis,* and *Margolisiella* (Mathur *et al*. 2020). Rhytidocystids are non-motile and, like gregarines, transmit passively by environmentally persistent oocysts. They also have a direct life cycle and some species are suggested to have intracellular life stages (Leander and Ramey 2006, Miroliubova *et al.* 2020). In comparison with the gregarines, most research on rhytidocystids has focused on resolving their phylogenetic position and

describing the species, and their effect on their hosts are known. However, *Rhytidocystis* infection has been reported to be common among hosts (all studied individuals infected) and the infection load has been described as high (Miroliubova *et al.* 2020).

1.3 Determinants of symbiont dynamics

Obligate symbionts cannot live without their host. All host organisms are potential habitats for their symbionts, but not all hosts always are infected and the infection patterns in natural populations often show high variability also spatially and temporally (Knowles *et al.* 2011, Poulin 2006). This variation is due to differences in encounter rates (Hansen *et al*. 2004, Karvonen *et al*. 2004.) and variation in host susceptibility or resistance to infection (Hassell and Anderson 1984, Klein 2004). Various biotic and abiotic factors affect the encounter rates, such as the variations in spatial and temporal co-occurrence between the parasite and the host, the avoidance behavior of the host and local environmental factors (e.g. Poulin 2013). Furthermore, hosts can also vary in their ability to tolerate or clear the infection. These variations lead to differences in symbiont transmission ability and fitness. In the following sections, the different biotic and abiotic factors affecting infection dynamics are discussed.

1.3.1 Host characteristics

Genetic diversity within individuals and populations is fundamental for species to evolve and persist with changing environmental conditions. It is also of central importance in mediating host-symbiont interactions because it promotes resilience against parasitic infections and enables coevolution between hosts and their symbionts (Bérénos *et al.* 2011, Gomulkiewicz *et al*. 2003, Ekroth *et al.* 2019). Host populations that have low genetic diversity are expected to be more vulnerable to infection than populations with greater diversity (Elton 1958, Garrett and Mundt 1999, Keesing *et al.* 2006, Hamilton 1987). High host population genetic diversity, on the other hand, is expected to lower the probability that a parasite encounters a susceptible host because more diverse populations are more likely to contain individuals that are resistant to infection (Anderson and May 1982, Keesing *et al*. 2006). Therefore, high genetic diversity in host populations can limit parasite transmission to a new generation of hosts (Altermatt and Ebert 2008, Ostfeld and Keesing 2012). At the individual level, a host's genetic composition can either increase or decrease susceptibility. Low genetic variation within individuals has been associated with greater susceptibility to infections (Whitehorn *et al.,* 2011, Kaunisto *et al*. 2013) and high genetic diversity has been associated with higher resistance in individual hosts (Isomursu *et al.* 2012, King and Lively 2012). For mutualistic symbionts, the effects of host genetic diversity on the interaction outcome are far less clear than for parasitic symbionts (Stoy *et al.* 2020). Because mutualistic interactions are

beneficial for both parties, maintaining them could be expected to include little genetic variation. However, high genetic diversity for both host and symbiont level has been detected in many mutualistic interactions (Boutin *et al.* 2014, Chong and Moran 2016, Simonsen and Stinchcombe 2014). It has been suggested that this diversity could be generated by environmental transmission of the symbionts, sexual recombination and horizontal gene transfer and that high diversity may be advantageous in maintaining mutualism (Stoy *et al.* 2020).

Host size and age are important characteristics in determining parasitic symbiont dynamics. Larger and older hosts have generally more parasites because larger hosts might be able to provide higher amount of resources and more space to support larger parasite communities (Kuris *et al.* 1980, Poulin 1995) or because they have had time to accumulate symbionts over time (Rohde 1994). Large size can also be connected to increased consumption rates or switches in the feeding behavior, which could further increase the encounter rates (Grunberg and Sukhdeo 2017, Taskinen and Valtonen 1995, Zelmer and Arai 1998). On the other hand, lager (and older) hosts might have less parasites due to developing resistance (or immunity) (Rohde 1994), or they might be in a better nutritional condition and therefore, may better tolerate parasitic infections (Miller and Cotter 2018). Sometimes host condition and body size have even stronger effects on host tolerance to parasitic infection than genetic composition. For example, host's survival during chytrid fungus infection was positively correlated with body mass in common toads while host heterozygosity was negatively correlated with survival (Smith *et al.* 2022)

Host population density is another factor that affects symbiont dynamics especially for parasitic symbionts. High host densities are expected to increase prevalence of infection as well as the infection loads (Anderson and May 1978, Arneberg *et al.* 1998) through increased encounter rates and more efficient symbiont transmission (Patterson and Ruckstuhl 2013, Rifkin *et al.* 2012). However, this is not always the case. The relationship between host density and parasitism can depend on many factors, such as the parasite's life history and environmental factors that affect the transmission. For directly transmitted parasites, especially with passive environmental transmission, the abundance is expected increase with host density (Anderson and May 1978). However, for actively (mobile) transmitted parasites, the encounter-dilution effect might reduce the infection load within infected hosts (Rifkin *et al.* 2012). Aquatic environments also provide exception to the traditional density-dependence of symbiont infections: because symbionts can accumulate in the environment locally if they are transported with water currents, their abundance can be independent of host density (Murray 2009).

1.3.2 Coinfections

Because more than half of all organisms on Earth are symbionts living in a close association with their hosts (de Meeûs and Renaud 2002), it is not surprising that most infections in nature consist of multiple symbiont species (de Meeûs

and Renaud 2002, Petney and Andrews 1998). Interactions between coinfecting symbionts within a single host can strongly affect their occurrence, distribution, and dynamics as well as their host populations (Clerc *et al*. 2019, Pedersen and Fenton 2007, Rovenholt and Tate 2022, Seabloom *et al*. 2015). The main ways coinfections can affect the dynamics of other symbionts are competition (Dobson 1985) and facilitation (Zélé *et al*. 2018). In a situation where the symbionts share the same resources (i.e., habitat and food) within their host, competition that results in a lower infection of the weaker competitor might occur. Interference competition can occur between species that infect the same physical space within the host (Pedersen and Fenton 2007). For example, coinfection of two intestinal worm species infecting lambs lead to a reduced reproductive output of the other due to lack of space to lay their eggs (Dobson and Barnes 1995). Resource competition (e.g., for food) can occur indirectly, when two species exploit the same host resource for their nutrition. For example, competition for host resources with a coinfecting virus reduced the replication and growth of another virus infecting the tea tortix (Ishii *et al*. 2002). Similarly, coinfection with intestinal gregarines was found to decrease reproduction of a nematode in cockroach hosts (Randall *et al.* 2013).

Facilitation in coinfections occurs when an infection of one symbiont provides conditions for another symbiont to infect the same host and it can result in higher population size (infection load) of one of the infecting symbionts (Behnke *et al*. 2009, Zélé *et al*. 2014, Zélé *et al*. 2018). Direct facilitation involves mechanical facilitation, for example, by providing infection routes for another symbiont. An example comes from a coinfection of ectoparasite *Argulus coregoni* and a bacterium *Flavobacterium columnare* infecting rainbow trout. Feeding action of *A. coregoni* disrupts the host fish skin and weakens the protective mucus layer and thus provides an infection route for the bacterium (Bandilla *et al*. 2006). Facilitation could occur also indirectly, for example, by suppression of the host immune system by one symbiont, which then provides means for another symbiont to infect the host. For example, in wild rabbits the suppression of immune system has been suggested to be caused by myxoma virus, which then facilitates higher infection of nematodes in coinfected hosts (Cattadori *et al*. 2007).

1.3.3 Abiotic determinants

Abiotic factors mainly affect the symbiont encounter rates but can influence susceptibility of the hosts as well. The main effect comes from the variation in transmission efficiency of the infective stages in different environmental conditions, such as season and the local environmental factors (Pietrock and Marcogliese 2003, Poulin 2013). In marine environments, symbiont transmission is more strongly affected by the environment than in terrestrial habitats (Murray 2009). This is mostly due to the open nature of the environment and characteristics of water as a media (McCallum *et al.* 2004). For example, tidal currents and high velocity in marine environments can increase environmentally transmitting symbiont prevalence locally due to enhanced

transmission (Alaliyat *et al*. 2019, Correia *et al*. 2021, Halliday-Isaac *et al*. 2021). On the other hand, in rivers and streams, a lower water flow has been connected to higher prevalence of symbionts with a direct life cycle (only one host) because of local accumulation (Barker and Cone 2000).

In marine ecosystems, salinity is one of the most important environmental factors because it strongly influences the distribution of organisms and the interactions between species (Montagna *et al*. 2002, 2008). Therefore, it is not surprising that salinity can also affect transmission of marine symbionts, especially in estuarine and brackish habitats that are subjected to changes in salinity (Haskin and Ford 1982, Studer and Poulin 2012). For example, several studies have shown that increased salinity increased the reproduction of trematode parasites in their first intermediate host (snail), which enhances transmission to their second host (amphipod) (Mouritsen 2002, Rees 1948, Studer and Poulin 2012). For dinoflagellates infecting blue crabs, low, unusual salinities caused their transmission stages (dinospores) to become inactive and thus halted their transmission completely (Coffey *et al*. 2012). Symbionts have also been suggested to be more resilient to salinity changes than their hosts, especially with additional environmental stress from rising temperatures (Kenigsberg *et al.* 2022). Besides the effects on transmission, salinity can also affect symbiont reproduction (Pisani *et al.* 2008, Soleng and Bakke 1997). Pisani *et al*. (2008) found that a ciliate symbiont infecting estuarine grass shrimps was able to reproduce more efficiently in higher salinities than where it was originally collected. In contrast, *Gyrodactylus salaris* infecting Atlantic salmon was found to reproduce equally in low salinity (brackish water) as in fresh water, but in marine salinities their population growth halted (Soleng and Bakke 1997).

Temperature is another key mediator of symbiont transmission in aquatic environments as even a mild change in water temperature can alter their transmission (Barber *et al*. 2016, Harvell *et al.* 2002). For example, higher water temperature was found to increase infection intensity of a trematode larvae in rainbow trout, most likely due to increased transmission (Gopko *et al.* 2020). Many symbionts develop faster in higher temperatures, which can lead to higher transmission (Kolman *et al*. 2015, Platonova and Palinauskas 2021). Additionally, higher than normal temperatures cause stress on animal hosts (Buckley and Huey 2016, Williams *et al*. 2016). Ectothermic organisms, such as the marine invertebrates, are especially sensitive to temperature stress because the rates of their biological and biochemical processes are strongly affected by temperature and can eventually lower their survival (Abram *et al*. 2017, Kern *et al*. 2015). Changes in temperature can further alter other abiotic factors, such as oxygen levels, and increase the harmful effect on aquatic animals (McBryan *et al.* 2013, Minuti et al. 2021). When a host is facing thermal stress, it can become more susceptible for infections due to temperature induced immunity suppression (Dittmar *et al.* 2014).

1.4 Determinants of symbiont diversity

For free-living organisms, species richness across ecosystems is dependent on the availability of suitable habitats, resources, and habitat heterogeneity (Jetz *et al*. 2009, Stein *et al*. 2014). Because hosts act as both habitats and resources for symbionts, host species richness is expected to determine symbionts richness (Hechinger and Lafferty 2005). This is especially evident for parasitic symbionts and the hypothesis, ''host diversity begets parasite diversity'', is increasingly supported by empirical evidence (Edwards and Vidrine 2020, Johnson *et al*. 2016, Kamiya *et al*. 2014, Thieltges *et al*. 2011) (Fig. 1A). Another factor influencing symbiont richness is the host specificity (Poulin *et al*. 2011). Highly host-specific symbionts typically infect only one host. In communities that have high species richness, the number of different hosts is also high. This could lead to an increase in the diversity of symbionts within the community (Poulin 1997). For example, studies have shown that host specific tick richness is positively correlated with their hosts' richness in different geographical areas (Esser *et al*. 2016, McCoy *et al*. 2013).

From the infection dynamics point of view, host community diversity also plays a role (Fig. 1B). Because a more diverse host community can include a greater proportion of unsuitable hosts (Thieltges *et al*. 2008), it is expected that higher host diversity could decrease the rate of parasite transmission and thus lower the prevalence and infection loads observed in host populations (dilution effect) (Keesing *et al*. 2006). Both laboratory and field studies have found evidence of a dilution effect in many parasite-host systems (Civitello *et al*. 2015 Johnson and Thieltges 2010, Johnson *et al*. 2013). For example, in a study conducted with deer mice and SNV virus, the authors found that increasing diversity in host communities led to lower prevalence of the virus since fewer intraspecific encounters between the host individuals limited virus transmission (Clay *et al*. 2009). Another example comes from the tick transmitted bacteria *Borrelia burgdorferi* in vertebrates, where dilution occurred via reduced transmission in diverse host communities and the host abundance was the main contributor to increased risk of infection (Halsey and Miller 2020). In contrast, a dilution effect was not observed in vector transmitted avian parasites and host and vector diversity (Ferraguti *et al*. 2021).

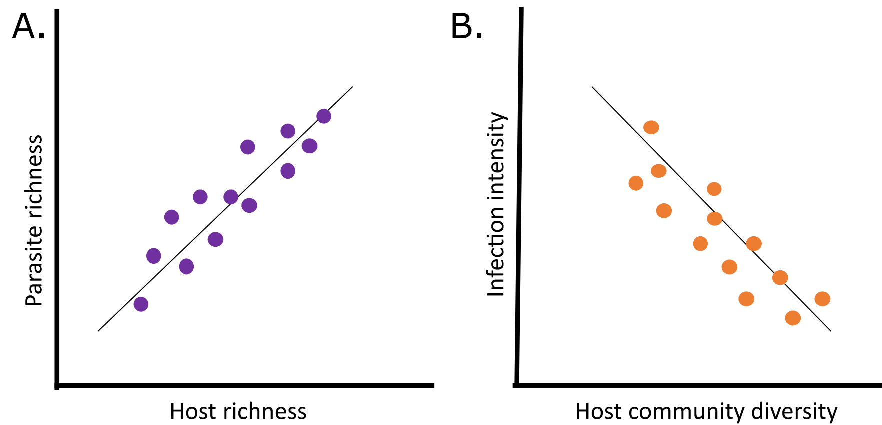

FIGURE 1 Schematic graphs illustrating the effects of host richness and community diversity on A. obligate symbiont species richness and B. infection intensity of a single symbionts.

1.5 Studying cryptic symbionts

Symbiotic interactions have been studied in multiple ways to understand the factors maintaining and limiting them. To understand the underlying mechanisms driving these relationships, experimental approaches to manipulate the interactions are often used (Richardson *et al.* 2012). However, in many cases experimental approaches are not feasible due to the characteristics of the symbionts or their hosts or when knowledge about the interacting species is limited. Symbionts can, for example be 'cryptic', i.e. difficult to detect from the tissue of their host or occur in low density (Baker and Romanksi 2007, Mieog *et al*. 2007). This is the case especially for aquatic obligate symbionts that cannot be cultured outside their hosts and for which experimental infections are difficult to carry out. While terrestrial insects can directly be fed the symbiont infective stages with their food (e.g Woltz *et al*. 2022), same can be difficult to conduct in water media. Additionally, symbiont free hosts are needed for experimental infections and such host cultures can be difficult to produce and maintain in a laboratory. A way to circumvent these issues is to directly observe hosts in natural conditions to obtain metrics that can be used to describe symbiont dynamics. Symbiont prevalence (the proportion of infected individuals) is a key variable in studying symbiont dynamics (Morand and Krasnov 2010). Prevalence is used to indicate the relative success of a symbiont in infecting a certain host population (Garcia-Langoria *et al.* 2019). Another metric commonly used is the infection load (or intensity of infection). This indicates the severity of infection or, in another words, how many symbionts infect a single host at a given time. Prevalence and mean infection load can be used to describe differences between host samples. Below, the use of different molecular methods is introduced for studying symbiont dynamics (prevalence and infection load) and diversity when traditional experimental approaches cannot be used.

1.5.1 Molecular methods

To obtain accurate estimates of symbiont dynamics, the use of molecular tools is particularly efficient (Sibley *et al.* 2012). Compared to traditional methods utilizing microscopy, molecular methods have several advantages including the efficiency to process a large number of samples quickly and with improved sensitivity over morphological characterization especially in host-symbiont systems with low infection intensity or coinfections of similar symbiont species (Maia *et al*. 2014, Mieog *et al.* 2007, Renoux *et al.* 2017). PCR methods are commonly used, in which specific genes from the target symbiont are amplified and detected from DNA samples. For example, real-time quantitative PCR (qPCR) in a common method used in quantifying symbionts from blood DNA samples of fish (Renoux *et al*. 2017), lizards (Maia *et al.* 2014) and tissue samples of corals (Mieog *et al*. 2007) and primates (Ludwig *et al*. 2021). For the use of molecular methods, species specific molecular markers are needed. Widely used molecular markers for species identification in eukaryotic organisms are the hypervariable regions in the small subunit rRNA genes (16S rRNA, 18S rRNA) and the mitochondrial genes (e.g., cytochrome b, mitochondrial control regions)(Patwardhan *et al.* 2014). One of the most used markers for species delimitation in animals is the cytochrome c oxidase I (*cox1*) (Hebert *et al.* 2003) and it has been shown to be useful in studying many eukaryotic taxa (Ogedengbe *et al*. 2011, Pawlowski *et al*. 2012).

Digital PCR (dPCR) is a third generation quantitative PCR method (Li *et al.* 2018). It is based on partitioning and randomly distributing the sample into small partitions prior to amplification, which makes it highly sensitive in detecting rare targets in low concentrations. (Hindson *et al*. 2011). Amplification occurs in each partition individually and the fraction of positive and negative partitions is analyzed using Poisson statistics to calculate the target concentration (Hindson *et al*. 2011, Sykes *et al*. 1992). Because of high resolution and increased sensitivity, dPCR has been commonly used in clinical studies, GMO detection, and food security (Demeke and Dobnik 2018, McMahon *et al.* 2017, Sedlak *et al.* 2014). For detection of symbionts that are unculturable outside their hosts, the dPCR is particularly useful since it gives the concentration of the target per reaction (absolute quantification) and does not require labor intensive standard curves.

Another useful molecular tool in symbiont studies is the utilization of high throughput sequencing (HTS) methods, which have enabled studies from whole microbiomes of hosts to the detection of symbiont infective stages from environment (eDNA)(Geisen *et al*. 2015, Lokmer and Wenger 2015, Pagenkopp Lohan et al. 2016, Reveillaud *et al.* 2014). HTS methods are especially useful in studying the diversity of environmentally transmitted symbionts and where they co-occur with their potential hosts (Singer *et al*. 2021). In fact, the recent increase in the use HTS has improved the understanding of diversity patterns of eukaryotic symbionts that have been traditionally difficult to identify and widened the knowledge of microbial diversity as a whole (del Campo *et al*. 2019, Mahé *et al*. 2017). Furthermore, HTS offers possibilities for the discovery

of new species from environmentally derived sequences (Hunter *et al.* 2018, Pawlowski *et al.* 2018).

1.6 Aims of the thesis

Interactions between obligate symbionts and their hosts (antagonistic, beneficial, and even neutral interactions) have been crucial in shaping evolution through generation of phenotypic and genetic diversity, which has further altered the physiology of their hosts and enabled species to widen their niches. In this thesis, different molecular methods were used to investigate the infection patterns of marine apicomplexan symbionts infecting the bristle worm, *Pygospio elegans*, the interactions involved in symbiont-symbiont relationships, as well as symbiont-host interactions and the ecological determinants of symbiont diversity on a large spatial scale. Monitoring the infection patterns in natural populations helps identifying the factors affecting these relationships and that are of great importance in understanding these interactions. Still, the outcomes of many of these interactions are unknown since knowledge about the symbionts themselves is lacking, which further makes it difficult to develop efficient tools for monitoring. Therefore, the first aim of the thesis was two-fold; to 1) develop a robust tool for detecting and quantifying cryptic unculturable symbionts from DNA samples extracted from the whole host organisms, and to 2) use this tool to quantify infection patterns of a marine apicomplexan (*Rhytidocystis* sp.) in natural host populations.

Because the majority of marine invertebrates, such as *P. elegans*, are of little commercial value, knowledge of their symbionts, and their interactions with these symbionts is scarce and has not been prioritized. However, benthic invertebrates have many crucial ecological roles in coastal habitats. Therefore, investigation of the apicomplexans infecting them can contribute to a better understanding of what drives or limits symbiotic interactions in marine environments. In addition, such investigations add to the knowledge on neglected biodiversity and on species interactions in these important benthic ecosystems, which are most vulnerable to anthropogenic impact (Chen 2021). The second aim of the thesis was to characterise the infection and coinfection patterns of two marine apicomplexans *Rhytidocystis* sp. and *Selenidium pygospionis* in natural host populations and to investigate the association of host genetic diversity on the infection.

Although apicomplexans are long known to be common and abundant in marine ecosystems, their diversity patterns in relation to the diversity of their hosts are understudied compared to apicomplexan and host diversity in terrestrial environments. The third aim of the thesis, therefore, was to fill the knowledge gap in this field of research and answer the question “Does marine apicomplexan species richness follow their hosts’ richness?”. In doing so, I also aimed to study whether the infection dynamics of apicomplexans are affected by host community diversity i.e., whether higher host diversity lowers the

infection intensity of an apicomplexan infecting *P. elegans* (through a dilution effect).

2 MATERIALS AND METHODS

2.1 Study organisms

2.1.1 The host, *Pygospio elegans*

The polychaete, *Pygospio elegans* Claparède, 1863, is a small tube-dwelling worm that inhabits sandy coastal habitats in marine and brackish waters. This species can occur in extremely high densities in these habitats (Bolam 2004, Bolam & Fernandes 2003, Thonig *et al*. 2016), and therefore play an important role in the function and diversity of benthic communities. For example, it is an important prey for other invertebrates and fish (de Vlas 1979, Mattila 1997) and consumes benthic diatoms and other smaller organisms from the sediment. This species lives inside sand tubes it constructs from the surrounding sand particles and while doing so, it actively participates in bioturbation of sediments. Bioturbation activities greatly influence community interactions in the benthos through effects on sediment oxygen concentrations, and consequently affect the biomass of organisms living there, the rate of organic matter decomposition, and the regeneration of nutrients needed for primary production (Reise 2002, Witte *et al.* 2003), further emphasizing the importance of polychaete species in benthic communities.

2.1.2 The symbionts

Pygospio elegans is infected by three apicomplexan symbionts: the archigregarine *Selenidium pygospionis* (Paskerova *et al*. 2018), the eugregarine *Polyrhabdina pygospionis* (Paskerova *et al*. 2021), and an undescribed marosporidan in the genus *Rhytidocystis*, from henceforth referred to as *Rhytidocystis* sp. All of the symbionts infect the worm's intestine but the nature of their interaction with their host is not known.

2.2 Sample collections

2.2.1 Host-symbiont interactions and apicomplexan infection dynamics (I + II + III)

In the first chapter, already collected DNA samples stored in -20°C were used to study the infection patterns and seasonal dynamics of *Rhytidocystis* sp. These samples were collected from four natural *P. elegans* populations from Lynæs, Lammefjord, Vellerup and Herslev in the Isefjord-Roskilde Fjord complex in Denmark (Thonig *et al.,* 2016) (Fig. 2A) seasonally from from March 2014 to February 2015. Sand tubes containing *P. elegans* were collected using a 1mm mesh sieve and sieving randomly sampled surface sediment collected from below water. Live worms were separated from their tubes under a dissecting microscope and sized (length in µm from eyespot to the beginning of gills). In addition, the population density and environmental variables (salinity, temperature, and sediment characteristics) were measured seasonally (see Thonig *et al.* 2016 for details). DNA was extracted from whole worms with DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol (Thonig *et al.* 2017). For this thesis, a subsample of the samples from the earlier study was used (19 to 28 hosts per population and sampling time).

For the second chapter, to study the coinfection patterns of *Rhytidocystis* sp. and *S. pygospionis*, and the association between host genetic diversity and the infection, fresh hosts were collected from two previously sampled populations in Denmark (Herslev and Vellerup) and from one population in Cramond Beach, Edinburgh, UK (55°58'N, 3°17'53W). All samples were collected in early November in 2018 and 2019 and the sampling procedure was the same as in the first chapter, except in Cramond Beach the sand tubes were collected from tidal pools during low tide. Forty (in 2018) and twenty (in 2019) live worms from each site were preserved individually in microtubes containing DNA/RNA Shield (Zymo Research) on site and transported to University of Jyväskylä. DNA was extracted following the same protocol as in the first chapter.

For the third chapter, to study how *Rhytidocystis* sp. infection is associated with host community richness, 24 individual *P. elegans* samples were collected from four sites across the North Sea and the Baltic Sea (List, Saltö, Herslev and Gollwitz)(Fig. 2B). Sand tubes containing the live worms were sampled as described above and worms separated from their tubes were stored in >96% EtOH in individual 1.5 ml microfuge tubes and transferred to University of Jyväskylä for DNA extraction.

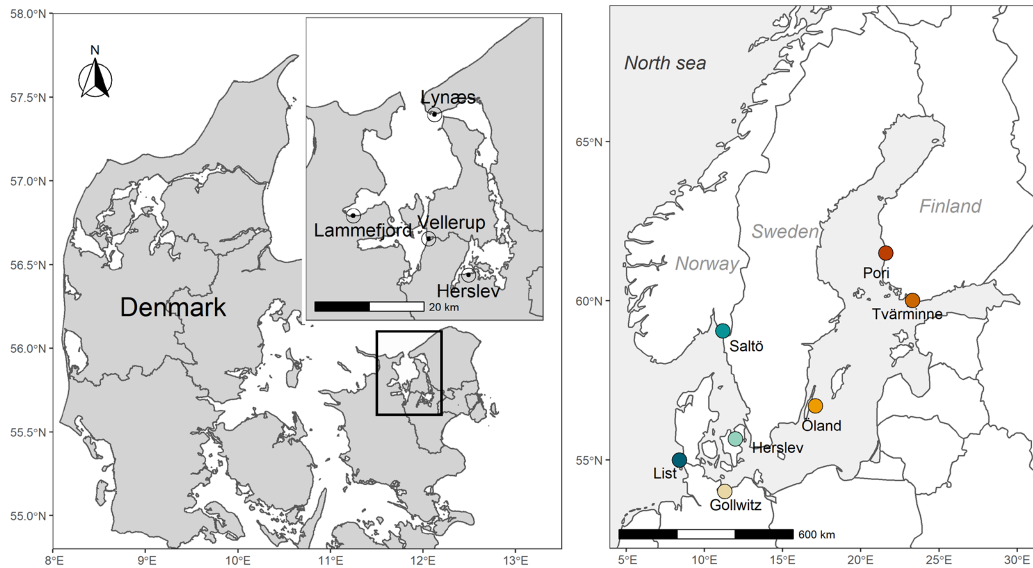

FIGURE 2 Sampling sites for the studies. A. Four sites in Isefjord-Roskilde Fjord (I, II) B. Baltic Sea sampling sites (III).

2.2.2 Apicomplexan richness in relation to benthic invertebrate richness (III)

For the third chapter, in order to investigate if the apicomplexan species richness patterns were associated with benthic invertebrate richness, samples from seven sites across the North Sea and Baltic Sea were collected in August 2018 (Fig. 2B). Apicomplexan richness was assessed from sediment as follows: from each site, hand-held corers (5 cm diameter, min. 15 cm depth) were used to collect sediment in three replicates at water depth 0-0.80 m, with the exception in Tvärminne where sampling was also performed by SCUBA at 3.8-5 m depth. From the top layer (approx. 5 mm) of each core, two scoops of sediment (two technical replicates) were taken with sterile razor blades avoiding the core sides and placed in 1.5 ml microcentrifuge tubes. Samples were preserved in 99% ETOH and kept frozen at -18°C until transport to University of Jyväskylä for DNA extraction. Unfortunately, not all samples were preserved well enough for the transfer and for those sites, technical replicates had to be used, meaning that two samples were taken from the same core and one from another core. These sites were Herslev, Gollwitz and Tvärminne.

To determine benthic invertebrate species richness, five replicate samples were collected at the proximity of the sediment samples using similar cores as described above (15 cm diameter, min 30 cm depth). Detailed species identification is described in Petersen (2021). In short, samples were sieved through a 1mm mesh, and the material that remained in the sieve was fixed in 99% EtOH on site. The animals were identified from the preserved material based on morphological characteristics using common identification keys (Barnes 1994, Hayward and Ryland 2017 Kirkegaard 1992a, 1992b) and taxonomic validity was confirmed in World Register of Marine Species (Horton *et al*. 2020).

2.3 Droplet digital PCR assay (I+II+III)

This assay was used in all three chapters of this thesis. The detailed descriptions of the different experiments for assay validation are presented in the first chapter, and here only the main steps are summarized. First, primers were designed to amplify the mitochondrial *cox1* gene of *Rhytidocystis* sp. and *S. pygospionis* (Table 1) using Primer3 software (https://primer3.ut.ee). Although *cox1* is not a single copy gene, it was chosen to target the apicomplexans since the sequences showed sufficient divergence from the host's *cox1* sequence and from each other. Additionally, the other gregarine known to infect the host, *P. pygopionis*, has lost *cox1* gene from its mitochondrial genome, thus, targeting *cox1* of *S. pygospionis* ensures that the assay does not amplify the nontarget gregarine.

TABLE 1 Primers sequences for *cox1* gene of the two apicomplexans, *Rhytidocystis* sp. and *Selenidium pygospionis* amplified with the ddPCR assay.

Species	Primer pair	Sequence	Target length	Chapter
***Rhytidocystis* sp.**	ApiCox1F	ACTGGTCTATCAAGTGTACTGGC	226 bp	I
	ApiCox1R	GATCACCACTAAATTCAGGGTCA		
Selenidium pygospionis	SelCox1F	ACAGGCTGTTGTCGGTCATT	200 bp	II
	SelCox1R	AGTCTGCTCTACGTCGAACA		

ddPCR was performed using Bio-Rad's QX200™ Droplet Digital™ PCR System. The reaction mix was prepared to a volume of 20 µl and 2 µl of the DNA templates were added to each reaction, the final reaction volume being 22 µl. Samples were partitioned into droplets with the QX200 Droplet Generator (Bio-Rad). The resulting droplets were transferred to a PCR plate and target genes were amplified using a PCR protocol validated for each primer pair. After the amplification, the end result was analyzed with Bio-Rad's Droplet Reader and QuantaSoft Analysis Pro software using default settings for ABS (absolute quantification) experiments. The ABS experiment calculates the concentration of the target in copies µl^{-1} of the final 1X ddPCR reaction. Therefore, the infection load (copies ng^{-1} total DNA) for each sample was calculated using the following formula:

$$C_{ng} = \left(\frac{C_{ddPCR} \times V_R}{V_S}\right) / \, C_{DNA}$$

where C_{ng} = concentration of *cox1* per ng of input DNA (ng µl^{-1}), C_{ddPCR} = concentration per reaction (copies µl^{-1}), V_R = volume of the reaction (µl), V_S = volume of the sample (µl) and C_{DNA} = concentration of the DNA sample (ng µl^{-1}.) Only reactions containing ≥ 10,000 droplets were included in downstream

analyses. Positive and negative reactions were separated by a manually set threshold in relation to the negative control in QuantaSoft Analysis Pro.

To produce positive controls for the assay, 88 *S. pygospionis* and 33 *P. pygospionis* individuals were isolated from hosts collected in September 2020 and provided by colleague Gita Paskerova (St. Petersburg State University, Russia). Gregarines were collected from dissected host intestines by micromanipulation, washed three times with filtered (Millipore 0.2 µm) seawater and transferred into microtubes, one for each species. The samples were pelleted with centrifugation to remove excess seawater and stored in 100 % EtOH for transport to University of Jyväskylä. EtOH was evaporated from the samples and the DNA was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Lucigen) following the manufacturer's instructions and eluted in 10 µl of EB buffer.

2.4 Microsatellite genotyping (II)

For studying if host genetic diversity is associated with the infection of the two marine apicomplexans, seven microsatellite loci were amplified from 18 to 36 hosts per site and year. Descriptions of the loci and amplification protocols can be found in Chapter II. In short, reactions were done using 1x Qiagen Multiplex PCR Master Mix and the resulting fragments were separated with ABI PRISM 3130xl and analyzed with GeneMapper® v.5 Software. For each sample, population genetic parameters, such as observed and expected heterozygosity (H_O and H_E), mean allelic richness (AR), inbreeding coefficient (F_{IS}) and pairwise differentiation among sites (F_{ST}) were calculated using GenAlEx v. 6.501 (Peakall & Smouse, 2012) plugin in Microsoft Excel (2016) and FSTAT v. 2.9.4 (Goudet, 1995). AR values were rarefied using the smallest sample size. Statistical testing for differences in F_{IS} was performed with 499 permutations and a significance level of $\alpha = 0.05$ was used. The statistical significance for pairwise F_{ST} values ($\alpha = 0.05$) was obtained with Bonferroni correction after 60 permutations in FSTAT. Comparison of H_O, H_E, AR, F_{IS} between populations and years was done with 10,000 permutations in FSTAT. Analysis of molecular variance (AMOVA) among sites was calculated with GenAlEx and the significance level of $\alpha = 0.05$ was obtained with 9999 permutations. Individual heterozygosity ranging from 0 = entirely homozygous to 1 = entirely heterozygous was calculated for each host individual by dividing the number of heterozygous loci with the total number of loci genotyped (Coltman *et al*. 1999).

2.5 Amplicon sequencing of eukaryotic 18S rRNA gene and diversity measures (III)

Apicomplexan species richness was determined with high throughput sequencing of eukaryotic 18S rRNA genes from the sediment samples. DNA was extracted from three replicate samples from each site and amplified with primers targeting non-metazoan 18S rRNA V4 region and the sequencing was done using Ion Torrent PGM with an Ion 318 Chip Kit version 2 (Ion Torrent, Life Technologies) internally at the University of Jyväskylä. Detailed descriptions on DNA extraction, the used primers and amplification protocols are found in the third chapter.

The single-end raw reads produced by the HTS were first demultiplexed by sample specific barcodes and sequencing adapters were removed with Cutadapt (version 1.18, Martin 2011). Reads were filtered to a minimum of 50 bp and maximum of 400 bp with Cutadapt and quality limits were defined as a minimum phred score of 20 for 80% of the bases in each read using FASTX (Hannon 2010). Reads were then dereplicated and filtered for singletons and chimeras with VSEARCH (version 2.15.1, Rognes *et al.* 2016). All reads were aligned to the Silva seed database (version 138, Yilmaz *et al.* 2014) using Mothur (version 1.44.3, Schloss *et al.* 2009) and clustered with using VSEARCH with 97% similarity threshold. Resulting operational taxonomic units (OTUs) were assigned to taxonomy using VSEARCH, at a similarity of 95-97.5% with the Protist Ribosomal Reference (PR2) database v4.1.14 (Guillou *et al.* 2013) using BLAST method with 80% similarity in Mothur. All OTUs assigned to Metazoa and Archaeplastida were removed, as well as unclassified taxa. OTUs assigned to unclassified Alveolates (n = 155) were manually blasted against NCBI nucleotide database and the accession numbers of best hits were checked against the PR2 database to correct their taxonomy when possible.

The diversity of apicomplexan species in each site was determined as observed number of OTUs and Shannon index (alpha diversity). Because metabarcoding studies are characterized by differences in read numbers between samples, alpha diversity estimates were rarefied by subsampling for the minimum number of reads by repeated random subsampling with 100 trials. Normality of the estimates was confirmed with Shapiro-Wilk normality test, and the differences between sites were tested with ANOVA. Apicomplexan beta diversity was estimated with Bray-Curtis dissimilarity index, visualized with nonmetric multidimensional ordination (NMDS) with k=3, and the community turnover (differentiation) was tested with PERMANOVA.

3 MAIN RESULTS AND DISCUSSION

3.1 ddPCR assay specificity and validity (I)

Molecular methods, such as PCR or HTS, are crucial in detecting symbionts when traditional methods are not sufficiently accurate (e.g. Refardt and Ebert 2006). Additionally, when the symbiont of interest is small, occurs in low density and is difficult to identify from the host tissue, using microscopy methods may overlook the symbiont load (Inácio da Silva *et al*. 2021, Refardt and Ebert 2006). Because ddPCR yields absolute concentration measures (copies μl^{-1}) and therefore does not require standard curves (Hindson *et al*. 2013), it is particularly useful for detecting these kinds of symbionts. In this chapter, the main goal was to develop a ddPCR assay specific to detect and estimate *Rhytidocystis* sp. infection dynamics in natural host populations.

Previous studies have shown that ddPCR is sensitive and reproducible in detecting symbionts from host DNA (Koepfli *et al*. 2016, Srisutham *et al*. 2017, Wilson *et al*. 2015). In this study, the ddPCR assay showed great linearity for 10-fold dilution series (Fig. 3) and the dynamic range experiment showed that it can detect symbionts at very low density (LoD was 0.86 copies μl^{-1}). Also, compared to qPCR which typically has LoD of 4 to 10 target molecules (Forootan *et al*. 2017, Rønning *et al*. 2003), the LoD of the developed assay was much lower. Additionally, variation in the target concentration (CV%) was small especially when concentration was high. Furthermore, the concentration of the target was independent of the droplet generation event and also not affected by the number of droplets in each reaction. These results showed that ddPCR can be effectively used in detecting and estimating infections of cryptic symbionts from DNA extracts of hosts especially in cases where the symbionts are difficult to extract to provide reference material needed to produce standard curves (Salipante and Jerome 2019).

While the detection of the symbiont by the developed assay was shown to be reliable, the quantification of infection load based on the *cox1* copy number does not indicate the exact infection load of *Rhytidocystis* sp. because a single individual can have multiple copies of this gene. For example, Leander and Ramey (2006) noted that *Rhytidocystis polygordiae* has multiple mitochondria. Currently, the copy number of *Rhytidocystis* sp. *cox1* is not known but it can be assumed to occur in multiple copies. Therefore, the used assay only yields relative infection load and in order to gain more realistic estimates, a single copy marker would be preferable. Despite this limitation, the ddPCR assay can be used to estimate the symbiont infection load, and thus allows further insight into the infection dynamics and biological interactions between the symbiont and its host, as well as other symbionts the host might be infected with. When hosts are known to be infected by multiple closely related symbionts, assay specificity is especially important. The assay specificity validation experiment showed that the primers designed to amplify *Rhytidocystis* sp. *cox1* did not target the other apicomplexan symbionts that might also be present within the hosts, *S. pygospionis* and *P. pygospionis* (Paskerova *et al.* 2018, 2021). Therefore, the assay not only enables detection of symbionts that are otherwise difficult to quantify, but it also enables studying coinfections of apicomplexan species across host populations efficiently (Chapter II).

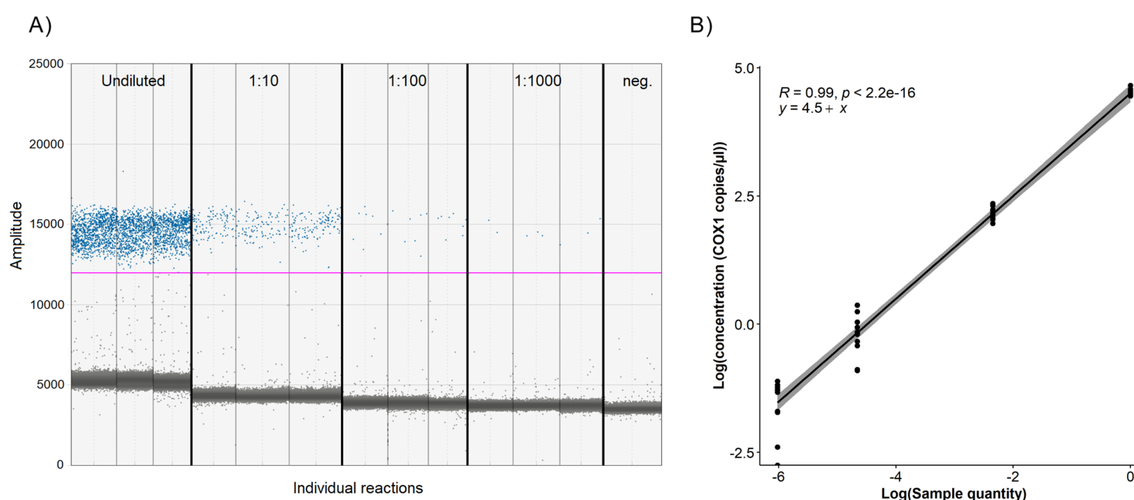

FIGURE 3 Sensitivity and precision for the assay. A) 1D ddPCR plot showing examples of 10-fold dilution series. The x -axis shows individual reactions in three replicates for the undiluted, 1:10, 1:100 and 1:1000 diluted concentrations and y -axis shows the amplitude of the fluorescence as relative fluorescence units (RFUs). Positive reactions (blue) and negative reactions (dark grey) are separated by a manually set threshold (pink line, at amplitude 11309). B) The sample quantity in log scale plotted against *cox1* concentration (copies μl^{-1} given by the ddPCR) in each replicate. Significant correlation coefficient between the absolute concentration (*cox1* copies μl^{-1}) and the ten replicates of each dilution shows good linearity.

3.2 Dynamics of *Rhytidocystis* sp. infection in four natural host populations (I)

Monitoring the patterns of infection can help to unravel the interactions between the symbiont and its host and shed light to the underlying mechanisms maintaining these interactions. The goal of this study was to demonstrate the use of the ddPCR assay for studying infection dynamics of a cryptic symbiont in natural host populations. Four seasonally sampled *P. elegans* populations from the Danish Isefjord-Roskilde fjord (Fig. 2A) were surveyed for *Rhytidocystis* sp. infection dynamics. Because host characteristics and environmental conditions can affect the transmission of the symbionts (Anderson and May 1978, Morand and Poulin 1998, Poulin 2013, Taskinen and Valtonen 1995) and therefore also the prevalence and infection loads, the second aim was to assess if host size and population density or environmental factors (organic content in the sediment) affected the prevalence of infection or the infection load.

The main finding of this study was that the proportion of infected hosts was overall very high (85.7%), but it varied spatially. The highest prevalence was found in Herslev, where almost all hosts were infected throughout the sampling period. In Lammefjord the prevalence was relatively low and ranged from 36% to 50%. Intermediate prevalence was detected in Lynaes, where the proportion of infected hosts ranged from 48% to 72%. Only in Vellerup, prevalence varied also temporally, from high (69.2 %) in March, to very low in May (18.5%), and increasing again towards fall so that 95% of hosts were infected in November. The infection load measured as the mean number of *cox1* copies ng^{-1} of host DNA varied spatially but also seasonally: the highest mean infection load was detected in Herslev and lowest in Lammefjord and it increased towards fall in all populations. In Vellerup, the highest infection load peaked earlier in August, but in the other populations it was highest in October (Lammefjord and Lynaes) and November (Herslev). Previous studies have found varying infection patterns, with some symbionts being more prevalent in spring (Kristmundsson *et al.* 2015), some showing similar patterns to *Rhytidocystis* sp. (Kirk *et al*. 2013) and some remaining constant regardless of the season (Halliday-Isaac *et al.* 2021). In a freshwater environment, varying seasonality of apicomplexan infection has also been found (Grunberg and Sukhdeo 2017).

For environmentally transmitted symbionts, such as most apicomplexans, host population density can increase the transmission and lead to a higher prevalence and infection load (Anderson and May 1978). For example, a blood apicomplexan infecting the damselfish around the US Virgin Islands was found to have higher prevalence when the host fish population density was also higher (Halliday-Isaac *et al*. 2021). However, in this study no association between host density and infection dynamics of *Rhytidocystis* sp. was found. A similar result was observed in a study of apicomplexans infecting freshwater

amphipods (Grunberg and Sukhdeo 2017). This could be due to environmental conditions related to the aquatic habitat that affect the symbiont transmission. For example, in aquatic environments water currents might move the environmentally transmitted infective stages and thus the transmission could be less affected by the density of host populations than in more stable environments (Murray 2009).

In addition to host population density, host body size can influence the infection patterns of symbionts (Morand and Poulin 1998). The results from this study showed that larger hosts were found to be more likely infected than smaller ones, but host size had not effect on the infection load. This could be, for example, caused by higher probability of encountering of the symbiont infective stages due to higher consumption rate of larger hosts. For example, in *Daphnia,* larger individuals often are more infected by microsporidian parasites due to higher feeding rates (Mangin *et al*. 1995, Stirnadel and Ebert 1997). In *P. elegans*, larger individuals are more prone to deposit feeding than filter feeding (Hentschel 1998), which could further increase the encounter rates of *Rhytidocystis* sp. infective stages deposited to the sediment. However, as the infection load did not increase with host size, this suggests that larger hosts, although more likely to be infected, might also be more resistant to the infection (Miller and Cotter 2018).

Finally, environmental characteristics could influence symbiont transmission and therefore prevalence and infection loads. Here, salinity, temperature and different sediment characteristics were measured in the four sites. Due to high collinearity between the variables, only organic content of the sediment was used to analyze the association between *Rhytidocystis* sp. infection and environmental factors. Organic content indicates how much nutrients are available for the host in the environment (Cheng *et al*. 1993) and in this study, it was found to be negatively associated with both prevalence and infection load, although the effect was not statistically significant. Previously, it has been shown that polychaetes living in a nutrient poor environment tend to increase their foraging area (Kihslinger and Woodin 2000). This could lead to a higher encounter with symbiont infective stages and explain the observed higher prevalence and infection loads when the organic content was low. Additionally, the results could indicate that hosts living in a habitat with higher organic content could be in a better nutritional condition and therefore be better able to resist infection.

Overall, the results of this study indicate that the infection dynamics of *Rhytidocystis* sp. vary spatially and to some degree also seasonally. Infection probability was affected by host size but not host population density. These results suggest that the relationship between *Rhytidocystis* sp. infection and its polychaete host are not straightforward and influenced by spatial and temporal variations in both host and symbiont biology and factors that affect symbiont transmission. This study is the first to describe the infection patterns of any marosporidan symbiont and research on other marine apicomplexan infection dynamics is also scarce. Therefore, these results add to the knowledge of understudied symbiont dynamics in natural host populations and provide

better understanding of the interaction of marine apicomplexans and their hosts.

3.3 Coinfection patterns of two marine apicomplexans and host genetic diversity (II)

Coinfections are common in nature (Petney and Andrews 1998) and one of the most important biotic factors affecting host-symbiont interactions (Rovenholt and Tate 2022). Because interactions among the coinfecting species (symbiont - symbiont interactions) can also greatly influence the outcome of the infection, studying coinfection patters in could help in disentangling these interactions. In this study, the main goal was to investigate the coinfection of two marine apicomplexan species, *Rhytidocystis* sp. and *Selenidium pygospionis,* infecting the polychaete worm, *P. elegans*, by quantifying their prevalence and infection loads in three host populations. Furthermore, because genetic diversity within individuals and populations is crucial for species to maintain their adaptability in the face of selection pressures, such as those caused by parasite infection, the second aim of this chapter was to determine the association between marine apicomplexan infection and host genetic diversity measured by microsatellite heterozygosity.

Coinfections were relatively common in the studied populations, as more than one third of all hosts were infected by both apicomplexans. However, there was a significant difference in the rate of coinfection between the populations. In Cramond Beach (Scotland), coinfections were more common than in Herslev and Vellerup (Denmark)(Fig. 4A). Additionally, all hosts were infected with either of the two symbionts in Cramond Beach, while 13% to 17% of hosts were found to be completely symbiont-free in Herslev and Vellerup, respectively. Almost all studied hosts that were infected by *S. pygospionis*, also had *Rhytidocystis* sp. infection and when both apicomplexans coinfected the same host, the *Rhytidocystis* sp. infection load was significantly higher compared to hosts without *S. pygospionis* infection (Fig. 4B). However, no significant change was found in *S. pygospionis* infection loads between coinfection and single infection. For aquatic gregarines, coinfections have previously been reported from many taxa (Grunberg and Sukhdeo 2017, Paskerova *et al*. 2018, 2021), but relatively little is known how the coinfecting symbionts affect each other. For example, coinfections by two gregarines infecting the freshwater amphipod, *Gammarus fasciatus*, were reported to be rare and show a negative interaction, when the presence of one gregarine lowered the abundance of the other, possibly due to competition, was detected (Grunberg and Sukhdeo 2017).

The observed coinfection patterns between *Rhytidocystis* sp. and *S. pygospionis* could be an indication of beneficial interaction through facilitation. Facilitation could occur indirectly by one symbiont suppressing the host

immune system, causing the host to be more susceptible to infection by another symbiont (Cattadori *et al.* 2007) or directly when infection by one symbiont creates routes for infection by the other (Bandilla *et al*. 2006). Coinfecting symbionts could, alternatively, show negative association with each other through competition (Clerc *et al*. 2019, Graham 2008, Ezenwa and Jolles 2011). The two symbionts both infect the intestine of *P. elegans* (Paskerova *et al*. 2018), which could lead to a negative outcome through competition of host resources (Dobson 1985, Dallas *et al.* 2019). However, as coinfections were frequent, occurred in all studied populations, and most importantly, *Rhytidocystis* sp. infection load was higher when *S. pygopsionis* was present, synergistic interactions might explain the observed patterns better.

In many cases, coinfections of two symbionts can vary temporally (Karvonen *et al*. 2019). This means that two symbionts are more likely to infect the same host sequentially rather than at the same time through temporal niche partitioning to avoid competition (Grunberg and Sukhdeo 2017). In Chapter I, *Rhytidocystis* sp. infection was found to be seasonally dynamic in the Danish host populations, with infection load being high in fall and declining in spring. However, whether infection by *S. pygospionis* shows seasonal dynamics is not known. If *S. pygospionis* has a different seasonal cycle, the observed differences in prevalence and infection loads could be an indication of temporal niche partitioning between the two species.

Finally, the infection patterns of both symbionts were independent of host genetic diversity. Moreover, host individual heterozygosity did not differ between hosts that had single species infection compared to coinfected hosts. Assuming that all apicomplexans are parasites, the expectation was that higher individual host heterozygosity would be connected to lower prevalence and infection loads. These results suggest that the differences in prevalence and infection loads are more likely due to differences in encounter rates, possibly caused by local environmental factors such as salinity (marine in Scotland and brackish in Denmark)(Coffey *et al*. 2012), which could be suboptimal for *S. pygospionis* in the brackish environment, or tidal currents (Halliday-Isaac *et al*. 2021), which could enhance transmission and thus prevalence in Cramond Beach. Furthermore, *S. pygospionis* has not been previously found in the Baltic Sea. Its low prevalence in the Danish populations might also reflect the geographical distance between the studied sites, and it is possible that *S. pygospionis* has not yet fully managed to colonize the Danish host populations.

In conclusion, coinfections were common, but their frequency differed between the sites (Scotland vs. Denmark). *Rhytidocystis* sp. showed a higher infection load when the host was infected also with *S. pygospionis,* which could be due to a synergistic effect caused *S. pygospionis* infection. Also, no association between coinfection or infection by individual apicomplexans and host genetic diversity either on individual or population level was found. These results suggest that differences in symbiont encounter rates in these sites might affect the infection dynamics more than differences in host susceptibility. As the nature of the interaction between the apicomplexans and their host is currently

not known (mutualistic or parasitic), it is possible that parasite-mediated selection pressure might not be the case in this system.

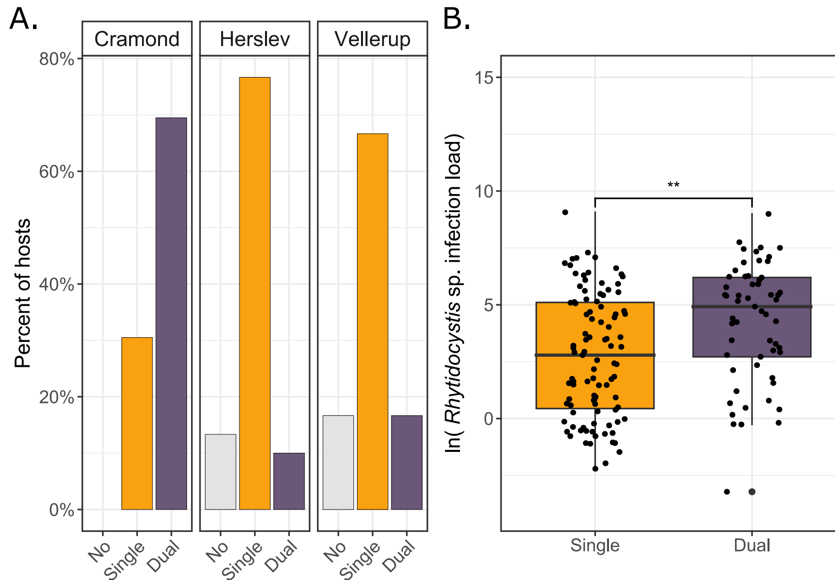

FIGURE 4 Coinfection patterns of *Rhytidocystis* sp. and *Selenidium pygospionis* in Cramond Beach and the Danish (Herslev and Vellerup) populations of *P. elegans*. **A.** The prevalence of single (orange) and dual (purple) infected hosts and hosts with no infection (light gray) in the whole dataset (n = 176). **B.** Coinfected hosts had higher *Rhytidocystis* sp. infection load compared to single-infected hosts. The solid line depicts the median and ** indicates significance level of $p < 0.01$.

3.4 Apicomplexan diversity correlations with their potential hosts along the Baltic Sea coastal communities (III)

Species richness for free-living organisms is strongly influenced by the availability of habitats and resources (Jetz *et al.*, 2009, Stein et al., 2014). For symbiotic organisms, hosts comprise both habitats and resources and therefore, host species richness is expected to determine the richness of symbionts, especially for parasitic organisms (Edwards and Vidrine 2020, Hechinger and Lafferty 2005, Johnson *et al*. 2016, Kamiya *et al.* 2014, Thieltges *et al*. 2011). The goal of this study was to describe the diversity of the marine Apicomplexa in the Baltic Sea coastal benthos and correlate it to the diversity of potential host species, the benthic invertebrates. The diversity of benthic invertebrates is known to decrease along the Baltic Sea salinity gradient from marine conditions at the opening to the North Sea towards almost freshwater at the Bothnian Bay and Gulf of Finland (Remane 1934, Zettler *et al*. 2014), and because marine apicomplexans are known to infect a wide variety of benthic invertebrates (especially mollusks and annelids)(e.g. Leander 2008), this system offers a great setting for studying apicomplexan diversity patterns related to their hosts.

The diversity of marine apicomplexans was determined using a metabarcoding approach targeting the apicomplexan infective stages in the benthos because the richness of apicomplexans in environmental samples has been suggested to correlate with the diversity of their hosts (Geisen *et al*. 2015, Mahé *et al*. 2017). In this study, alpha diversity of apicomplexan species richness decreased towards lower salinity (Fig. 5A), however, the effect was not significant. Additionally, a weak nonsignificant positive association between apicomplexan and host richness was found (Fig. 5B). The most marine site (List) had both highest host and apicomplexan richness, and the richness of both groups decreased towards sites with lower salinity. Previously, only a few studies conducted in terrestrial environments have investigated apicomplexan richness association with their hosts. For example, apicomplexan and host richness determined from eDNA metabarcoding was found to be positively correlated in alpine meadows (Singer *et al*. 2020). In another study, Geisen *et al.* (2015) observed a high relative abundance of potentially parasitic protists (Apicomplexa and others) from soil samples in sites dominated by earthworms (potential hosts), but the authors did not test any correlations.

Several factors could explain why the association between apicomplexan richness was only weakly positive in this study. Firstly, apicomplexan infections have not been characterized from most of the benthic invertebrates occurring in the Baltic Sea and it is possible that they are not hosts to these symbionts. However, the lack of knowledge on apicomplexan infections in these hosts is most likely due to insufficient sampling and general lack of research on apicomplexan diversity in brackish water systems (del Campo *et al*. 2019). Additionally, benthic invertebrate symbiont fauna is generally less studied due to low economic importance of these animals.

A more likely explanation could be the unexpected diversity decline of hosts observed in sites with intermediate salinity (Saltö and Herslev). As noted by Remane (1936) and several studies since then, the lowest richness of benthic animals should be found in sites with salinity ranging from 5 to 7 ppt (Tvärminne and Pori in this study) when both marine and freshwater species richness' are low. However, both diversity and richness of benthic invertebrates decreased significantly from the highest salinity site (List, salinity 33 ppt) to medium salinity (Saltö 26 ppt and Herslev 15 ppt) and either increased again (Shannon index) or remained the same (species richness) towards the lower salinities. This observation could be due to a temperature induced oxygen depletion in Saltö and Herslev (Ritter and Montagna 1999, Smyth and Elliot 2016). In summer 2018, after which the sampling was conducted, water temperatures in the Baltic Sea were reported to be the warmest since 1990 (Siegel and Gerth 2019). This was pronounced especially at Herslev, where the temperature was almost 5°C higher than the normal average. Hypoxia tolerance of aquatic animals is reduced in higher temperatures (McBryan *et al*. 2013) and temperature tolerance can be lowered further in hypoxic conditions (Anttila *et al*. 2015, Vaquer-Sunyer and Duarte 2011, Verberk *et al*. 2013). Apicomplexan oocysts in the sediment might be less susceptible to short term environmental stress, and therefore not show such a drastic decrease in diversity. Furthermore,

increased temperature has also been connected to faster life cycle and increased reproduction in many symbionts (Clopton and Janovy 1993, Overstreet and Lotz 2016), which could have resulted in accumulations of the infective stages detected by metabarcoding, despite the decrease in host diversity.

Finally, the beta diversity of hosts showed a clear turnover along the salinity gradient, possibly due to a switch in species composition from marine to freshwater species (Zettler *et al*. 2014). Marine protists communities are generally expected to cluster distinctly from freshwater protists (Burki *et al*. 2021, Singer *et al*. 2021) and because apicomplexans are thought to be host specific, it could be expected that they would show similar clustering to their hosts. However, this was not the case in this study: PERMANOVA analysis showed differentiation in clustering, but there was more overlap among the least saline sites and among the sites within the Baltic Sea transition zone (Saltö and Herslev). These results, together with the richness patterns mentioned above, could be due to limitations associated with eDNA sampling methods (e.g., Burki *et al.* 2021). For example, the sediment samples are only a small snapshot of what is actually there and to obtain a better estimate, more replications (both true and technical replicates) should be used. In this study, the sediment samples used for apicomplexan detection were taken from separate cores than those used to sample the host species. The distribution of benthic animals tends to be patchy (Bolam, 2004, Bolam and Fernandes 2003, Thonig *et al*. 2016) and consequently, so could the distribution of apicomplexan oocysts. Therefore, it is possible that the full diversity of the symbionts was not captured with this sampling scheme.

In conclusion, marine apicomplexans are distributed across the Baltic Sea and their richness patterns indicate a possible positive association with their putative hosts. However, unexpected decrease in richness of the hosts, possibly due to environmental stress, and limitations in sampling could have masked a stronger positive correlation.

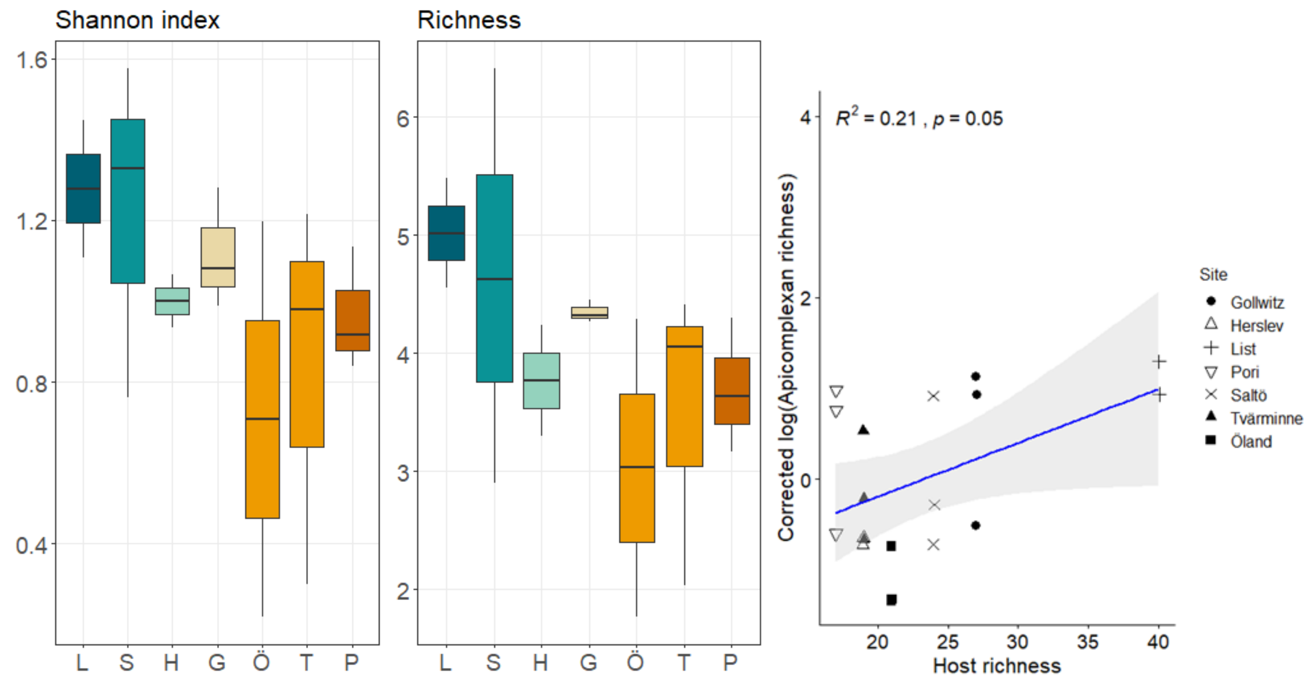

FIGURE 5 A. Alpha diversity estimated with Shannon index and observed OTUs/species richness for Apicomplexans (rarefied data, 29 OTUs, 13 reads per sample). L; List, S; Saltö, H; Herslev, G; Gollwitz, Ö; Öland, T; Tvärminne, P; Pori. B. Association between the richness of apicomplexan OTUs (corrected for read number variation) and the total richness of their hosts in each site. The blue line shows the slope of the linear regression and grey area notes the 95% confidence interval. Apicomplexan richness (the number of OTUs) was log transformed to fit the model assumptions.

3.5 Dilution effect in infection by *Rhytidocystis* sp. (III)

High host community richness could lower the prevalence and infection loads of a given symbiont through a dilution effect caused by a high number of unsuitable hosts species limiting symbiont transmission to susceptible hosts (Keesing *et al*. 2006). To investigate if dilution effect affects the infection patterns of a marine apicomplexan, *Rhytidocystis sp*., infection dynamics (prevalence and infection load) were measured from the host, *Pygospio elegans*, in benthic species assemblages sampled from four sites in the North Sea and the Baltic Sea (List, Saltö, Herslev and Gollwitz, see Fig. 2B) and correlated against benthic invertebrate diversity parameters (richness and abundance) in these sites. Richness was defined as the number of species observed in the area and abundance as the number of individuals of each species.

The prevalence of *Rhytidocystis* sp. infection was high in all studied sites and it did not differ between the sites. However, the infection load was found to be significantly higher in Herslev than in the other sites. When comparing infection loads to species richness and species abundance in the benthic invertebrate assemblages, signs of possible dilution effect were found. *Rhytidocystis* sp. infection load was positively correlated with the abundance of benthic invertebrate individuals. Additionally, the infection load was lower when the number of benthic invertebrate species was higher, although the effect was not significant. The fact that the effect of invertebrate abundance was stronger than the negative association with host species richness suggests that abundance is more important in determining *Rhytidocystis* sp. infection. For environmentally transmitted symbionts, dilution effects occur via limiting transmission either through reduced encounter rates or by limiting the density of the most suitable host, situations more likely to occur in host communities that have high species richness (Keesing *et al*. 2006). *Rhytidocystis* sp. is a host specific symbiont that transmits via infectious oocysts stages in the environment, where host polychaetes ingest them while foraging for food. In this study, host density was not measured. Therefore, it is difficult to disentangle whether susceptible host regulation or encounter reduction are the mechanisms behind the possible dilution effect observed. However, in Chapter I, *Rhytidocystis* sp. infection load was found to be independent of host density. Furthermore, it has been suggested that dilution through encounter reduction can reduce infection in host populations irrespective of host density (Civitello *et al.* 2015), as found for SNV virus infecting deer mice (Clay *et al*. 2009).

It is also important to keep in mind that the difference in infection loads between the sites could be a result of other factors and not only species richness or abundance. In Herslev, where highest prevalence and infection loads were observed, the low diversity of host animals could be due to stressful conditions around the sampling time (high temperature and possible hypoxia, see section 3.4.). Generally, species that face stress from suboptimal conditions are more

sensitive to infections (e.g., Oppliger *et al.* 1998). Therefore, the observed higher infection loads could be due to a lowered resistance in heat stressed hosts.

To conclude, *Rhytidocystis* sp. infection in coastal benthic communities of the Baltic Sea might be driven by dilution effect. These results indicate that the benthic invertebrate communities that have higher species diversity are more resilient to infections caused by apicomplexan symbionts. Therefore, species assemblages in coastal environments in the Baltic Sea that have low diversity could potentially be threatened by higher infection of apicomplexans. Combined with the high degree of anthropogenic stress in these habitats, this could further promote loss of biodiversity in this environment.

4 CONCLUDING REMARKS AND FUTURE DIRECTIONS

Recently, the question about the nature of gregarine infection (parasitic of mutualistic) has gained increasing interest (Rueckert *et al*. 2019), but very little research has focused on marine gregarines. Furthermore, the studies included in this thesis are first to focus the infection patterns of rhytidocystids. Therefore, the results of this thesis give novel information on infection dynamics and diversity of these understudied marine apicomplexans that infect benthic invertebrates. The results give indication of the biotic and abiotic factors affecting apicomplexan interactions both with each other and their hosts. Additionally, the results provide a novel tool for studying cryptic symbiont infection patterns when experimental approaches are not feasible. However, still much remains to be solved.

Firstly, the infection patterns of *Rhytidocystis* sp. and *Selenidium pygospionis* were found to vary spatially. It is important to resolve what drives these patterns of variation in prevalence and infection loads. For example, in Chapter II, *S. pygospionis* was found to be significantly less prevalent than *Rhytidocystis* sp. in the Danish host populations and, as discussed above, several factors could affect the observed differences. One way to approach this could be to perform experiments with controlled environmental settings (varying salinity and temperature), experimentally infect hosts from the Danish populations with *S. pygospionis* and monitor the infection success to find out if *S. pygospionis* is limited by one of the variables. However, since infected hosts cannot be distinguished from symbiont-free hosts without dissection, establishing a symbiont-free control group might be difficult. Another, perhaps more executable way, would be to investigate if the differences are caused by differences in symbiont seasonality. The sampling was conducted during November when *Rhytidocystis* sp. infection was known to be high in Denmark (Chapter I). However, it is possible that *S. pygospionis* follows a different seasonal cycle. Temporal niche partitioning is a phenomenon when two symbionts that have similar ecological requirements have evolved to infect the same host at different times to avoid competition (Kube *et al*. 2002, Sousa 1993).

This has been suggested to be the underlying cause for seasonal differences in gregarines infecting the amphipod *Gammarus fasciatus* (Grunberg and Sukhdeo 2017). Using the ddPCR assay, seasonal infection patterns of *S. pygospionis* could be estimated from the same samples that were used in Chapter I.

In Chapter II, an indication of facilitation occurring between *Rhytidocystis* sp. and *S. pygospionis* was found. *Rhytidocystis* sp. infection load was significantly higher when the host was also infected by *S. pygospionis*. However, similar pattern was not detected when comparing host infected with only *S. pygospionis* and coinfected hosts. This would again require utilization of experimental setups, where different factors could be controlled. For example, the fitness of each symbiont could be measured from host feces after coinfection experiments where hosts are infected with both symbionts and hosts that are infected with only one. Another reason for higher *Rhytidocystis* sp. infection loads during coinfections could be caused by so-called 'priority effects', where the order at which symbionts infect their hosts influences the intensity of the infection in a positive manner (Clay *et al.* 2019a, Clay *et al.* 2019b, Lohr *et al*. 2010). It would be interesting to study if this is the case in these apicomplexans, but again, it would be required to be tested experimentally.

In addition to the two apicomplexans studied in the chapters of this thesis, the host is known to be infected by a third symbiont, an eugregarine *Polyrhabdina pygospionis* (Paskerova *et al*. 2021). Interestingly, this symbiont has been found in coinfections with *S. pygospionis*, and shown higher infection loads compared to single infections, similar to *Rhytidocystis* sp. (Paskerova *et al*. 2018, Paskerova *et al*. 2021). These observations together with the findings from this thesis suggest that *S. pygospionis* could indeed act as facilitator for other infections. Adding *P. pygospionis* to the investigation of these apicomplexans' infection patterns could help to disentangle the interactions of these coinfecting apicomplexans within their host.

Finally, in Chapter III, the richness of apicomplexan species was found to be mildly positively associated with the richness of their putative hosts, the benthic invertebrates. The richness patterns were assessed with both molecular methods (apicomplexans from eDNA) and traditional methods based on morphological characteristics (benthic invertebrates). The richness patterns could also have been tested with different approaches. First, both hosts and apicomplexans could have been quantified from eDNA using primers designed to target eukaryotic organisms more widely (Singer *et al*. 2020). In this approach, the observed richness of both species groups in each sample could have been compared directly with each other. This approach could have also allowed deeper identification of the invertebrates (Yamamoto *et al*. 2017, Valentini *et al.* 2016). Alternatively, the apicomplexan richness could have been determined from microbiomes of a subgroup of the collected host animals instead of detecting from sediment. As noted above, the distribution of apicomplexan oocysts that were targeted with the eDNA survey, could be patchy within the sampled sites and the true richness might not be captured. Assessing the richness directly from the hosts could give more realistic indications of their diversity (Russell *et al.* 2018).

In conclusion, although the question about the ecological role (parasites, mutualists, or something in between) of the studied marine apicomplexans remains unsolved, investigating their infection patterns in natural conditions can contribute to a better understanding of the factors driving these interactions. Molecular tools, such as the ddPCR and HTS methods used in this thesis are efficient in detecting and monitoring apicomplexan symbionts in natural surroundings. However, as stated earlier, these interactions are difficult, if not impossible, to disentangle completely without controlled experimental approaches, which themselves can be challenging to execute. Given the ecological importance of benthic marine invertebrates (reviewed in Chen 2021), and the fact that apicomplexan infections might be parasitic to these animals, future studies are needed.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Symbionttien ja isäntien väliset vuorovaikutukset heikosti tunnetussa, oletettavasti loisivassa lajiryhmässä: merissä esiintyvät Apicomplexa -suvun symbiontit

Symbioosilla tarkoitetaan kahden erilaisen eliön läheistä yhteiseloa. Symbioottiset vuorovaikutukset isännän ja symbiontin välillä kattavat kaikki vuorovaikutukset loisimisesta (vahingollinen toiselle, hyödyllinen toiselle) mutualistisiin (molempia hyödyttävä) ja neutraaleihin vuorovaikutuksiin. Nämä vuorovaikutukset ovat olleet perustavanlaatuisessa asemassa eliöiden ekologisen ja evolutiivisten historian aikana. Symbioosi on vaikuttanut eliöiden fysiologiaan, käyttäytymiseen ja lajien välisiin toisiin vuorovaikutuksiin (peto-saalis -suhteet, kilpailu) sekä mahdollistanut lajien leviämisen uusille alueille. Symbioottiset vuorovaikutukset ovatkin olleet tärkeässä asemassa lajiutumisen ja sitä kautta koko maapallon luonnon monimuotoisuuden aikaansaamisessa. Tutkimukset ovat osoittaneet, että monissa tapauksissa symbioosilla ei kuitenkaan välttämättä ole havaittavaa vaikutusta kumpaankaan vuorovaikutuksessa olevaan osapuoleen. Symbioottiset vuorovaikutukset ovat usein myös dynaamisia ja ympäristöolosuhteiden muutokset voivat laukaista muuten neutraalin tai hyödyllisen symbioosin muuttumisen haitalliseksi (loisinta). Näiden vuorovaikutusten moninaisuuden ja niitä ylläpitävien tekijöiden tunteminen on tärkeää, jotta lajien sopeutumiskykyä muuttuviin elinolosuhteisiin voidaan ymmärtää.

Loisimisella tarkoitetaan vuorovaikutusta, jolloin organismi elää isäntänsä kustannuksella ja aiheuttaa yleensä jonkin verran haittaa isäntäeliön kelpoisuudelle (antagonistinen vuorovaikutus). Loisen aiheuttama infektio määräytyy pääasiassa kahdella tekijällä. Ensin loisten on kohdattava isäntä, jonka jälkeen isännän alttius tartunnalle määrittää, saako isäntä tartunnan vai ei. Alttius tietylle loiselle voi vaihdella suuresti yksilöiden välillä sillä siihen vaikuttavat useat eri tekijät. Tällaisia tekijöitä ovat esimerkiksi sekä isännän että loisen geneettinen monimuotoisuus, isännän sukupuoli ja ikä sekä ravitsemustila.

Apikomplexa -sukuun kuuluvat eliöt ovat monipuolinen ryhmä yksisoluisia eukaryootteja, jotka ovat yleisiä selkärankaisten ja selkärangattomien lajien symbiontteja. Ryhmää on pidetty yksinomaan loisivana, sillä siihen kuuluu monia patogeenisiä sekä lääketieteellisesti ja taloudellisesti tärkeitä lajeja, kuten malarian ja toksoplasmoosin aiheuttajia. Koska näiden symbionttien tutkimus on pääasiassa keskittynyt edellä mainittuihin taudinaiheuttajiin, niiden todellista monimuotoisuutta ei vielä tunneta. Tämä pätee erityisesti meriympäristöissä, joissa nämä symbiontit infektoivat monia pohjaelämiä, kuten nilviäisiä ja nivelmatoja, mutta niiden vaikutusta isäntänsä kelpoisuuteen ei juurikaan tunneta. Syitä tähän on monia; Apicomplexa -symbionttien pieni koko ja kryptisyys, eli niitä on vaikea havaita isännän kudoksesta, tai niiden infektiotiheys on alhainen. Symbioottisia vuorovaikutuksia tutkitaan usein kokeellisilla menetelmillä ja nämä lähestymistavat eivät kuitenkaan aina ole mahdollisia edellä mainituista

syistä. Tällöin molekylaariset menetelmät, kuten PCR-menetelmät ja DNA-sekvensointi ovat usein tehokkaampia ja tarkempia.

Tässä väitöskirjassa tarkasteltiin kahden heikosti tunnetun Apicomplexa -suvun symbiontin, *Rhytidocystis* sp. ja *Selenidium pygospionis*, vuorovaikutusta niiden isäntänä toimivan hiekkaputkimadon (*Pygospio elegans*) kanssa, sekä symbionttien välisiä vuorovaikutuksia. Tutkimuksessa analysoitiin symbionttien infektiotehokkuutta ja sen määrää luonnollisissa isäntäpopulaatioissa, sekä mitkä abioottiset ja bioottiset tekijät vaikuttavat infektion määrän vaihteluun. Tämän lisäksi tutkimuksessa määritettiin Apicomplexa -suvun symbionttien monimuotoisuutta suhteessa niiden isäntälajien monimuotoisuuteen Itämeren pohjaeläinyhteisöissä. Tutkimuksen tavoitteena oli selvittää, vaikuttaako eliöyhteisön monimuotisuus symbionttien infektion määrään isäntälajin yksilöissä. Tutkimus koostuu kolmesta kappaleesta, jotka on eritelty seuraavaksi.

Ensimmäisessä kappaleessa tavoitteena oli kehittää DNA-menetelmä, jolla voidaan havaita ja määrittää kryptisten symbionttien infektion määrää ja havainnollistaa sen käyttö luonnollisissa isäntäpopulaatioissa. Tutkimuksessa kehitettiin ddPCR menetelmä, jolla pystyttiin tarkasti ja luotettavasti tutkimaan *Rhytidocystis* sp. symbiontin infektion dynamiikkaa ja vuodenaikaisvaihtelua neljässä hiekkaputkimatopopulaatiossa. Tulokset osoittivat, että symbionttien infektion määrä vaihtelee paikallisesti ja infektiotehokkuus oli korkeinta loppusyksynä. Lisäksi tutkimuksessa havaittiin, että suurempikokoiset isännät saivat todennäköisemmin infektion kuin pienemmät, mutta isäntien populaatiotiheydellä ei ollut vaikutusta infektion tasoon. Nämä erot johtuvat mahdollisesti paikallista ympäristötekijöistä, jotka vaikuttavat symbionttien leviämiseen eli transmissioon.

Toisessa kappaleessa tutkittiin *Rhytodocystis* sp. symbiontin ja *S. pygospionis* symbiontin välistä vuorovaikutusta koinfektiossa, eli kun molemmat symbiontit infektoivat samaa isäntäyksilöä. Lisäksi tutkittiin, vaikuttaako isännän geneettinen monimuotoisuus infektion todennäköisyyteen, eli ovatko geneettisesti monimuotoiset yksilöt vähemmän alttiita näiden symbionttien aiheuttamalle infektiolle. Tutkimuksessa hyödynnettiin ensimmäisessä kappaleessa kehitettyä DNA-menetelmää symbionttien havaitsemisessa. Tulokset osoittivat, että koinfektiot olivat suhteellisen yleisiä tutkituissa populaatioissa, sillä yli kolmanneksella isännistä löytyi molempia symbiontteja. Lisäksi havaittiin, että *Rhytidocystis* sp. symbiontin infektion taso oli suurempi, kun myös *S. pygospionis* infektoi samaa isäntää, mikä viittaisi mahdolliseen fasilitaatioon eli hyödylliseen vuorovaikutukseen. Isännän geneettisellä monimuotoisuudella puolestaan ei ollut vaikutusta kummankaan symbiontin infektioon.

Viimeisessä kappaleessa tutkittiin Apicomplexa -suvun symbionttien esiintyvyyttä ja monimuotoisuutta Itämeren pohjaeläin yhteisöissä, sekä *Rhytidocystis* sp. symbiontin infektion määrää suhteessa eliöyhteisön monimuotoisuuteen. Näytteitä kerättiin koko Itämeren alueelta aina merellisitä olosuhteista (List, Pohjanmerellä) melkein makean veden olosuhteisiin (Pori, Pohjanlahdella). Tutkimuksessa havaittiin, että Apicomplexa -suvun symbiontteja esiintyy koko Itämeren alueella. Korkea lajien monimuotisuus isäntäpopulaation

elinympäristössä puolestaan vaikutti alentavan *Rhytidocystis* sp. symbiontin infektiotehokkuutta, mahdollisen diluutioefektin vaikutuksesta.

Tämä väitöskirjatutkimus osoitti, että Apicomplexa -suvun symbiontit ovat yleisiä Itämeren pohjaeläinyhteisöissä ja niiden infektion taso vaihtelee paikallisesti ja ajallisesti luonnollisissa isäntäpopulaatioissa. Tutkimus antaa uutta tietoa tekijöistä, jotka vaikuttavat näiden heikosti tunnettujen symbionttien esiintymiseen ja infektiotehokkuuteen, sekä esittää keinoja, jolla näitä vuorovaikutuksia voidaan tutkia tarkemmin. Kuten yllä on mainittu, symbioottisten vuorovaikutusten tutkiminen luonnossa ilman kokeellisia menetelmiä on erittäin haastavaa ja kokeellisten menetelmien kehittäminen onkin tärkeä seuraava askel selvitettäessä niiden laatua.

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ORIGINAL PAPERS

I

DROPLET DIGITAL PCR AS A TOOL FOR INVESTIGATING DYNAMICS OF CRYPTIC SYMBIONTS

by

Anna-Lotta Hiillos, Anne Thonig & Karelyn Emily Knott, 2021

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RESEARCH ARTICLE

Droplet digital PCR as a tool for investigating dynamics of cryptic symbionts

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Abstract

Interactions among symbiotic organisms and their hosts are major drivers of ecological and evolutionary processes. Monitoring the infection patterns among natural populations and identifying factors affecting these interactions are critical for understanding symbiont–host relationships. However, many of these interactions remain understudied since the knowledge about the symbiont species is lacking, which hinders the development of appropriate tools. In this study, we developed a digital droplet PCR (ddPCR) assay based on apicomplexan COX1 gene to detect an undescribed agamococcidian symbiont. We show that the method gives precise and reproducible results and enables detecting cryptic symbionts in low target concentration. We further exemplify the assay's use to survey seasonally sampled natural host (*Pygospio elegans*) populations for symbiont infection dynamics. We found that symbiont prevalence differs spatially but does not show seasonal changes. Infection load differed between populations and was low in spring and significantly increased towards fall in all populations. We also found that the symbiont prevalence is affected by host length and population density. Larger hosts were more likely to be infected, and high host densities were found to have a lower probability of infection. The observed variations could be due to characteristics of both symbiont and host biology, especially the seasonal variation in encounter rates. Our findings show that the developed ddPCR assay is a robust tool for detecting undescribed symbionts that are otherwise difficult to quantify, enabling further insight into the impact cryptic symbionts have on their hosts.

KEYWORDS
apicomplexa, cryptic symbiosis, droplet digital PCR, infection dynamics

1 | INTRODUCTION

Interactions between symbiotic organisms and their hosts dramatically influence not only organismal ecology and evolution, but also the dynamics of entire ecosystems (Faust & Raes, 2012; Godfrey-Smith, 2015). For the majority of symbioses, however, we do not know how the interacting species affect each other, much less the broader consequences of the symbiosis for communities or ecosystems. Often, it is the lack of tools for effectively monitoring or investigating symbiotic interactions that prohibits progress. In addition, a lack of general knowledge

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about the symbiotic organisms can limit the development of these tools (Pawlowski et al., 2012), leaving much of the functional and species-level diversity understudied.

Apicomplexans are a diverse group of microbial eukaryotes that include some of the most studied parasites with significant social and economic consequences (e.g., the genera *Plasmodium*, *Toxoplasma*, *Eimeria*, and *Cryptosporidium*) (Seeber & Steinfelder, 2016). Infecting majority of vertebrates and invertebrates in terrestrial and aquatic environments, apicomplexan species diversity is estimated to be over a million (Pawlowski et al., 2012). However, the bulk of apicomplexan diversity, especially in marine environments, is largely undescribed and contains many cryptic species (Janouškovec et al., 2015; Xavier et al., 2018). Additionally, despite the fact that some groups are known to be common and prevalent symbionts of marine invertebrates, little is known about the nature of their interactions (mutualistic or parasitic) with their hosts (Rueckert et al., 2019). Studying symbiont dynamics, even when the symbiont is cryptic, could help to shed light on the nature of their interaction with the host and their effect on host populations.

The presence of apicomplexans within host individuals and populations is traditionally determined with microscopy, but this can be difficult and labor intensive when studying large samples and may overlook small or cryptic microbial eukaryotes. In such cases, the use of molecular tools, such as PCR methods or amplicon sequencing, often are more appropriate. For instance, real-time quantitative PCR (qPCR) has been widely used in detecting and quantifying protistan parasites (e.g., Maia et al., 2014). Digital PCR (dPCR) is a third generation PCR method for quantification of target molecules in a nucleic acid sample (Li et al., 2018). It is based on partitioning and randomly distributing the sample into small partitions before PCR amplification, which takes place in each partition separately (Hindson et al., 2011). After amplification, the end-point reaction is visualized to determine the fraction of positive partitions (Hindson et al., 2011, 2013). From this fraction, the concentration of the target can be estimated using Poisson statistics (Sykeset al., 1992). In droplet digital PCR (ddPCR), the sample is partitioned into up to 20,000-nl-sized droplets by water–oil emulsion and the target concentration is therefore determined from the fraction of positive droplets. The advantage of partitioning lies in increased resolution and sensitivity; hence, ddPCR applications have been widely used in clinical studies, GMO detection, and food security (Demeke & Dobnik, 2018; McMahon et al., 2017; Sedlak et al., 2014).

Compared with traditional real-time qPCR, ddPCR has several major advantages. For example, it has higher precision and day-to-day reproducibility, especially when targeting rare copies (Brys et al., 2020; Doi et al., 2015; Hindson et al., 2013). ddPCR assays also tolerate inhibition better than qPCR (Dingle et al., 2013; Poh et al., 2020; Rački et al., 2014), and the end-point measurement of the nucleic acid quantitation is not affected by amplification efficiency. In addition, ddPCR does not require standard curves (Volgenstein & Kinzler, 1999). Therefore, the concentration of the target copies is an absolute measurement that is not reliant on Cq values. Furthermore, there is increased statistical power of ddPCR over qPCR (Taylor et al., 2017). These advantages make the method particularly applicable for molecular identification of symbionts. The increase of studies using ddPCR for quantifying haemoprotozoan infections (e.g., *Plasmodium* and *Babesia*) (Koepfli et al., 2016; Srisutham et al., 2017; Wilsonet al., 2015) and for detecting parasites from environmental samples (Mulero et al., 2020; Rusch et al., 2018) has already shown that the method is robust, reproducible, and capable of detecting rare copies of target DNA.

Despite the aforementioned advantages, ddPCR has not been used to its full potential, in particular for investigating cryptic symbionts and their ecological interactions with hosts. In this study, we show that ddPCR can shed light on biological interactions with precise estimates of cryptic, unculturable symbionts in natural host populations, indicating that the method is useful in molecular ecology research. We introduce a detailed ddPCR protocol and demonstrate its use by investigating the seasonal dynamics of an undescribed cryptic agamococcidian (Apicomplexa) infection for the first time in four polychaete (*Pygospio elegans*; Claparède, 1863) populations. By using specific primers, our assay targets the mitochondrial cytochrome oxidase *c* subunit 1 gene (COX1) of the agamococcidian symbiont and can be used effectively to determine symbiont prevalence and infection load from whole host DNA extracts.

2 | MATERIALS AND METHODS

2.1 | Study organisms

The host species, *P. elegans*, is a small polychaete worm that inhabits sandy coastal habitats throughout the northern hemisphere. This species can be a dominant member of benthic communities (Bolam, 2004; Bolam & Fernandes, 2003) and is an important prey item for other invertebrates and fish (Mattila, 1997). *Pygospio elegans* is known to host at least two apicomplexan symbionts: the archigregarine *Selenidium pygospionis* (Paskerova et al., 2018) and the eugregarine *Polyrhabdina pygospionis* (Paskerova et al., 2021), which both inhabit the worm's intestine. In our previous study of the host's transcriptome (Heikkinen et al., 2017), we detected the presence of a third apicomplexan symbiont. Based on 18S rDNA sequence similarity, this symbiont is most likely an agamococcidian (Order Agamococcidiorida; Levine, 1976), but its location within the host and definitive identification is not yet known. Agamococcidians are a small group of coccidian-like symbionts currently comprising two monogeneric families Gemmocystidae and Rhytidocystidae (Levine, 1976; Upton & Peters, 1986), but the taxonomy of the group has been questioned (Janouškovec et al., 2019; Mathur et al., 2020). Rhytidocystidae includes species infecting midguts of marine polychaetes (Leander & Ramey, 2006; Miroliubova et al., 2020; Rueckert & Leander, 2009), and some have been suggested to have intracellular life stages at least at an early stage of development within the hosts (Miroliubova et al., 2020). Because research on this group has mostly focused on resolving their phylogenetic position and

describing the species, the nature of their interaction with their hosts has not been studied previously.

2.2 | ddPCR assay specificity

Our goal was to produce an assay that could be used to detect and estimate infection loads of a cryptic symbiont from total DNA extracts (containing a mixture of host and symbiont DNA). A limited dataset of potential genes was available from our previous transcriptome study of the host (Heikkinen et al., 2017). We chose to design primers to amplify the presumed mitochondrial COX1 gene of the undescribed agamococcidian, as it showed sufficient divergence from the host's COX1 sequence. Primer3 software (https://primer3.ut.ee) was used to choose appropriate primers: forward primer ApiCox1F (5′-ACT GGT CTA TCA AGT GTA CTG GC-3′) and reverse primer ApiCox1R (5′-GAT CAC CAC TAA ATT CAG GGT CA-3′) to amplify 226 bp of the target gene. Amplicons were identical in sequence to the previously obtained transcript and showed high similarity to transcripts obtained from *Rhytidocystis* sp. ex. *Travisia forbesii* (Supporting information 1). Amplification efficiency of the primers was estimated to be 94.3% (slope = −3.465, r^2 = .997) using a qPCR. To demonstrate the specificity of the designed primers, the assay was checked against other apicomplexan DNA that might be present in the host gut; *S. pygospionis* (Archigregarinorida) (Paskerova et al., 2018) and *P. pygospionis* (Eugregarinorida) (Paskerova et al., 2021). Individual *S. pygospionis* (n = 88) and *P. pygospionis* (n = 33) cells were isolated from *P. elegans* collected in September 2020 (St. Petersburg, Russia). Non-attached gregarines were hand-picked from dissected host intestines by micromanipulation and washed three times with filtered (Millipore 0.2 μm) seawater. The cells were then transferred into microtubes, pelleted down with centrifugation to remove excess water, and stored in 100% EtOH for transport to our laboratory. Prior to DNA extraction, EtOH was evaporated from the samples and the DNA was extracted using MasterPure™ Complete DNA and RNA Purification Kit (Lucigen) following the manufacturer's protocol, except for the elution step, which was done in 10 μl of EB buffer. DNA concentration was checked using a Qubit 4.0 Fluorometer with 1X dsDNA HS Assay (Thermo Fisher Scientific).

2.3 | ddPCR protocol: reproducibility, dynamic range, and the limit of detection

ddPCR was performed using Bio-Rad's QX200™ Droplet Digital™ PCR System. Reproducibility of the assay was inspected with four replicates of three DNA samples: host with high (approx. 100 copies/μl), intermediate (approx. 50 copies/μl), and low (approx. 2 copies/μl) infection load. Because samples are partitioned into droplets in sets of eight in the QX200 system, two replicates of each sample were used in two different droplet generation events (four replicates for each sample). To inspect the dynamic range of the assay and the limit of detection (LOD), a 10-fold dilution series (1, 1:10, 1:100, and 1:1000) of the DNA extracted from the host with high infection load was performed in 10 replicates. Linearity over the dynamic range was determined by the coefficient of correlation r^2, calculated on the target concentration (copies/μl) measured in the dilution series replicates. Assay repeatability was determined by the % coefficient of variation (%CV = concentration standard deviation/concentration mean * 100) between the replicates. LOD is defined as the lowest target copy number in a sample that can reliably be detected. In this study, LOD was determined as the lowest concentration level for which all 10 replicates resulted in at least three positive droplets per replicate. In all experiments, a negative control containing nucleotide free water was used. The reaction mix was prepared to a volume of 20 μl per sample. We used 2X QX200™ ddPCR™ EvaGreen® (Bio-Rad) reagent mix. Primers were added to the mix in 1 μM together with 4.6 μl of sterile water. The reaction mix was divided into individual 0.5 ml microfuge tubes, and 2 μl of the DNA templates (varying concentration) was added to each tube so that the final reaction volume was 22 μl. Samples were partitioned into nanoliter-sized droplets with the QX200 Droplet Generator (Bio-Rad) using single-use DG8 cartridges and Droplet Generation Oil (Bio-Rad). Twenty microliters of each reaction mix was loaded to the cartridge, and the emulsion was made with 70 μl of oil. The resulting droplets were manually transferred with a multichannel pipet to a ddPCR™ 96-well PCR plate (Bio-Rad), which was heat-sealed with a foil cover.

The droplets were then subjected to thermocycling using a Bio-Rad C1000 thermocycler with a ramp rate of 2°C/s in each step. Initial denaturation of the DNA was done at 95°C for 3 min, after which the denaturation, primer annealing, and target extension steps were repeated for 40 cycles. The denaturation step was done at 95°C for 30 s, annealing temperature for the primers was optimized at 58°C for 1 min, and the target extension step was done at 72°C for 2 min. After the cycles, a signal stabilization step from 5 min at 4°C to 5 min at 90°C was added. Following the amplification, the droplets were immediately read with Bio-Rad's Droplet Reader.

2.4 | ddPCR data analysis

Absolute quantification of target gene copies was done with default ABS settings in QuantaSoft Analysis Pro 2.0 software (Bio-Rad). The ABS experiment estimates the concentration of the target in copies per microliter of the final 1X ddPCR reaction. Because one droplet can harbor one or more copies of the target, or none, the target concentration (copies/μl) is estimated by the software by calculating the mean copies per partition (λ) following Equation (1), where n is the total number of accepted droplets and k is the number of positive droplets counted.

$$\lambda = -\ln\left(1 - \frac{k}{n}\right) \tag{1}$$

The infection load (copies/ng total DNA) was calculated using the reaction mix volume (22 μl), the sample volume (2 μl), and the concentration (ng/μl) of the DNA sample following Equation (2), where C_{ng} is the number of copies per nanogram of total DNA, C_{ddPCR} is the reaction concentration (copies/μl) given by QuantaSoft Analysis Pro, V_r is the reaction mix volume, V_s is the sample volume, and C_{DNA} is the concentration (ng/μl) of total DNA sample.

$$C_{ng} = \left(\frac{C_{ddPCR} \times V_r\ (\mu l)}{V_s\ (\mu l)} \right) / C_{DNA} \tag{2}$$

Only reactions that had ≥10,000 droplets were included in further analyses. A threshold to separate the target positive and negative droplets was manually set in relation to the negative control by visual inspection.

2.5 | Survey of host populations

To determine the dynamics of the symbiotic infection in natural host populations, we quantified the agamococcidian COX1 gene copies in DNA extracts from whole *P. elegans* individuals collected from four populations in the Isefjord-Roskilde Fjord estuary at Lynæs, Lammefjord, Vellerup, and Herslev, Denmark (Figure 1). The populations were sampled seasonally in a previous study from March 2014 to February 2015 to describe the reproductive dynamics of the host (see Thonig et al., 2016, for sampling details). In addition, the authors determined size (length in micrometer from eyespot to the beginning of gills) for each host and population density (individuals in square meter). Environmental parameters (salinity, temperature, and sediment characteristics) were also collected seasonally (see Thonig et al., 2016).

DNA was extracted from whole host individuals using DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol for animal tissue and stored in −20°C (Thonig et al., 2017), and DNA concentration was measured with Qubit 4.0 Fluorometer with 1X dsDNA HS Assay (Thermo Fisher Scientific). In the current study, a subset of those samples was analyzed with ddPCR (Table 1). These sampling times were chosen for quantification of agamococcidian infection due to population specific changes in host reproduction and density patterns observed in these months (Thonig et al., 2016).

We used the ddPCR protocol described earlier to detect the cryptic symbiont (prevalence) and estimate its abundance (infection load). Prevalence of the symbiont in the host population was measured as the proportion of infected *P. elegans* individuals in the sampled population. An individual host was considered infected if more than 0 copies of the target gene per ng of total DNA were detected with the ddPCR assay. Infection load was defined as the number of COX1 gene copies/ng of total DNA, as in Equation (2), and analyzed with only infected hosts. Aggregation of the infection was inspected by calculating variance to mean ratios.

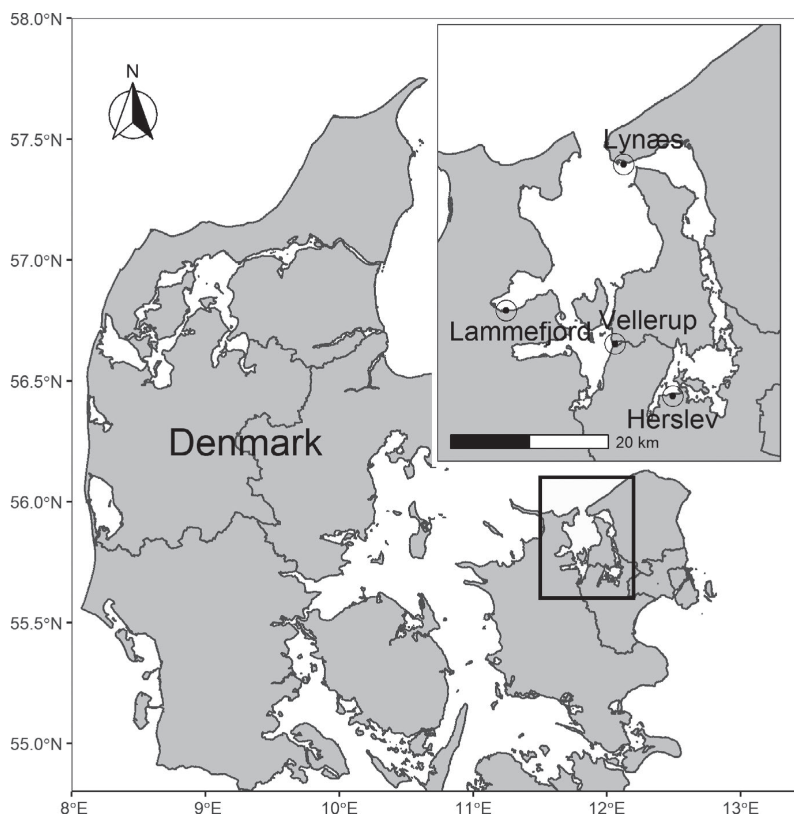

FIGURE 1 Location of the four sampling sites in Isefjord-Roskilde Fjord complex, Denmark

2.6 | Statistical analyses

In the ddPCR assay reproducibility experiment, the normality of the data was checked with Shapiro–Wilk test. The concentration did not follow a normal distribution; hence, a non-parametric Wilcoxon rank sum test was used to compare the replicates of high, intermediate, and low infection load medians between the two droplet generation events. The linearity of dynamic range was determined using least squares regression analysis of the linear relationship between sample quantity (fold) and COX1 concentration (copies/μl) in each dilution series replicates. The correlation of droplet count and the absolute concentration was analyzed for all replicates used in the two experiments described earlier ($n = 51$) with Pearson's correlation.

To avoid multicollinearity issues caused by significant correlations between predictor variables in the infection dynamics analysis, the correlations between the environmental variables and host density were inspected with Pearson's correlation. Organic content % in the sediment was chosen for further analysis, and all other environmental variables were excluded because of high correlations (Table 2). The prevalence of infection in the different populations and months was analyzed by logistic regression using population, sampling month, host length (in micrometer), host density (individuals in square meter), and the organic content % in the sediment as predictors. The log-transformed infection load was analyzed using linear regression with the same predictor variables. As the host density and the environmental variables were not measured in October, and two hosts did not have the length measurement, the missing values were imputed using Multivariate Imputation with Chained Equations method (mice) (van Buuren & Groothuis-Oudshoorn, 2011). In total for 99 out of 499 hosts (19.8%), there was no density or organic content measurements in October. We used multiple imputation to create and analyze five multiply imputed datasets. Incomplete variables (length and organic content) were imputed under fully conditional specification, using the predictive mean matching method and 50 iterations per imputation. The parameters were estimated in each imputed dataset separately and combined using Rubin's rules (Rubin, 1987). Each imputation was inspected visually by comparing the original data and the imputed values in a strip plot, and convergence of the iterations was inspected using Markov Chain Monte Carlo typed algorithm. The missing host density values were imputed for each population with linear estimation using the observed densities before (August) and after (November) the missing cases in October. The best fitting model was chosen with likelihood-ratio test (D3 in mice package) for logistic regression and with Wald test (D1mice package) for linear regression. For comparison, we also performed the analysis using only the samples with complete environmental data (excluding samples from October). All statistical analyses were performed in RStudio version 3.6.1 (05/07/2019) (https://cran.rproject.org).

TABLE 1 Number of host individuals in each population and month studied for agamococcidian infection dynamics

	Herslev	Lammefjord	Vellerup	Lynæs
March	27	25	26	25
May	28	25	27	25
August	25	25	25	25
October	23	25	26	25
November	28	24	22	19

3 | RESULTS

3.1 | Specificity, reproducibility, and detection limit of the ddPCR assay

Our ddPCR assay revealed on average 87.8 copies/μl (SE =3.35) of agamococcidian COX1 gene for the sample with high infection load (replicates 1a–d). The results showed little variability between the two droplet generation events for the same samples with average concentrations of 88.7 copies/μl in cartridge 1 and 86.1 copies/μl in cartridge 2 (Table 3), % coefficient of variation (%CV) being 8.8 and 9.5, respectively (Figure 2). For the sample with intermediate

TABLE 2 Pearson correlation coefficients (below diagonal) and the significance of the correlations (above diagonal) for environmental variables and host density

	Density	Mean salinity	Mean T	Mean C/N	Organic content	Water content	Porosity	Sorting φ	Median φ
Density	–	***	**	***	0.290	***	***	0.470	***
Mean Salinity	−0.25	–	***	***	***	0.079	***	***	0.096
Mean T	0.15	−0.2	–	***	0.077	***	***	*	***
Mean C/N	−0.24	0.19	−0.43	–	***	***	***	***	0.801
Organic content	0.05	0.4	0.09	−0.27	–	***	***	**	***
Water content	−0.39	0.09	−0.26	0.26	0.2	–	***	***	0.841
Porosity	−0.36	0.28	−0.19	0.27	0.38	0.9	–	***	0.297
Sorting φ	0.04	0.21	0.1	0.48	−0.15	−0.55	−0.46	–	0.822
Median φ	−0.22	−0.09	0.21	0.01	−0.29	−0.01	0.06	0.01	–

Note: Signif. *$p < .05$. **$p < .01$. ***$p < .001$.

TABLE 3 Concentration of COX1 (copies/μl) in reproducibility experiment ddPCR reactions (95% Poisson CI), mean concentration in each group and standard error of the mean (SE), % coefficient of variation (%CV) in each group, number of accepted droplets, mean amplitude of positive reactions fluorescence, mean amplitude of negative reactions fluorescence, and copies per partition (λ)

Sample	Copies/μl (95% CI)	Mean number of copies (SE)	%CV	Droplets	Mean amplitude of positives	Mean amplitude of negatives	λ
High_a	83.2 (78.1–88.4)			14,522	17,024	5604	0.071
High_b	94.2 (88.9–99.6)			15,489	16,819	5569	0.080
High_c	80.3 (75.2–85.5)			14,140	16,703	5190	0.068
High_d	91.9 (86.7–97.1)	87.8 (3.35)	6.6	15,815	16,474	5130	0.078
Intermediate_a	46.6 (42.9–50.3)			15,638	16,599	5284	0.040
Intermediate_b	49.5 (45.7–53.4)			15,379	16,761	5293	0.042
Intermediate_c	53.3 (49.3–57.3)			15,127	16,477	4913	0.045
Intermediate_d	49.6 (45.7–53.5)	49.8 (1.2)	4.8	15,211	16,608	4881	0.042
Low_a	1.86 (1.2–2.74)			14,527	16,867	5348	0.002
Low_b	2.77 (1.92–3.86)			13,584	17,252	5379	0.002
Low_c	1.96 (1.27–2.85)			14,451	16,716	4890	0.002
Low_d	1.22 (0.69–1.98)	1.95 (0.28)	28.2	13,490	17,150	4921	0.001
S. pygospionis	0	–		15,015	0	4701	–
P. pygospionis	0	–		16,931	0	4354	–
Neg. 1	0	–		16,049	0	4669	–
Neg. 2	0	–		17,322	0	4321	–

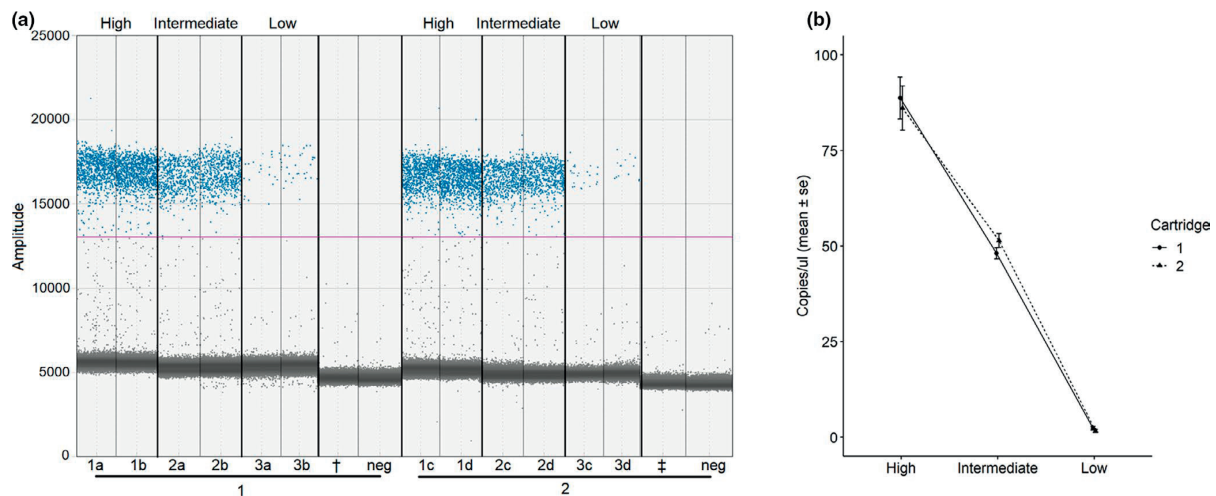

FIGURE 2 The ddPCR assay for hosts with high, intermediate, and low infection load of the agamococcidian symbiont. (a) 1D ddPCR plot. Replicates a and b contain droplets generated in cartridge 1, and replicates c and d contain droplets generated in cartridge 2. Individual reactions that are shown in the *x*-axis and *y*-axis show the amplitude of the fluorescence. Positive reactions (blue) and negative reactions (grey) are separated by a manually set threshold (pink line, at amplitude 13,033). Negative controls (neg) did not show any amplification above the threshold (concentration 0 copies/μl). The other apicomplexans possibly present in the mixed DNA samples (†*Selenidium pygospionis* and ‡*Polyrhabdina pygospionis*) also showed no amplification. (b) Concentration of agamococcidian COX1 gene copies per μl (mean copies/μl ± SE) in the two droplet generation events, cartridge 1 shown in solid line and black circle, and cartridge 2 shown in dashed line and black triangle

infection load (replicates 2a–d), the mean number of copies/μl was 49.8 (SE =1.2), ranging from 46.6 to 53.0 (Figure 2). The accuracy of the concentration measurements was highest in intermediate infection group, where %CV was 4.3 in cartridge 1 and 5.1 in cartridge 2 (Table 3). The mean number of copies/μl for the sample with low infection load was 1.95 (SE =0.28), being 2.32 copies/μl in cartridge 1 and 1.59 copies/μl in cartridge 2. %CV was 27.7 for low infection sample replicates ran in cartridge 1 and 32.9 in cartridge 2. Overall,

TABLE 4 Concentration of COX1 (copies/μl) in 10-fold dilution experiment ddPCR reactions (95% Poisson CI), mean concentration in each group and standard error of the mean (SE), %CV, number of accepted droplets, number of positive droplets, mean number of positive droplets, and number of copies per partition (λ)

Sample	Copies/μl (95% CI)	Mean number of copies (SE)	%CV	Droplets	Positive droplets	Mean number of positive droplets (SE)	λ
*1:1 Rep1**	*74.2 (62.3–86.2)*			*2422*	*148*		*0.063*
1:1 Rep2	88.6 (82.4–95.0)			10,485	761		0.075
1:1 Rep3	97.6 (916–104.0)			12,917	1028		0.083
1:1 Rep4	90.4 (84.0–96.8)			10,440	772		0.077
1:1 Rep5	88.3 (82.1–94.5)			10,874	786		0.075
1:1 Rep6	86.0 (79.9–92.2)			10,762	759		0.073
1:1 Rep7	95.4 (89.4–101.0)			12,396	966		0.081
1:1 Rep8	93.1 (87.1–99.1)			12,302	936		0.079
1:1 Rep9	86.0 (79.9–92.1)			10,811	762		0.073
1:1 Rep10	105.0 (99.0–111.0)	92.27 (1.964)*	6.4*	14,650	1249	891 (297)*	0.089
1:10 Rep1	10.5 (8.6–12.5)			12,695	113		0.009
1:10 Rep2	7.83 (6.3–9.4)			15,226	101		0.007
1:10 Rep3	10.1 (8.3–11.8)			15,248	130		0.009
1:10 Rep4	8.34 (6.9–9.8)			17,553	124		0.007
1:10 Rep5	9.33 (7.7–11.0)			15,321	121		0.008
1:10 Rep6	7.86 (6.4–9.6)			13,965	93		0.007
1:10 Rep7	7.14 (5.6–9.0)			11,404	69		0.006
1:10 Rep8	8.64 (6.9–10.6)			11,477	84		0.007
1:10 Rep9	9.11 (7.4–11.0)			12,831	99		0.008
1:10 Rep10	7.72 (6.0–9.7)	8.66 (0.33)	11.9	10,239	67	100.1 (31.7)	0.007
1:100 Rep1	1.04 (0.52–1.84)			11,272	10		0.0009
1:100 Rep2	0.41 (0.12–0.96)			11,613	4		0.0002
1:100 Rep3	0.41 (0.12–0.97)			11,414	4		0.0004
1:100 Rep4	0.82 (0.39–1.49)			12,886	9		0.0007
1:100 Rep5	1.28 (0.70–2.10)			12,001	13		0.0010
1:100 Rep6	0.71 (0.3–1.39)			11,554	7		0.0006
1:100 Rep7	0.66 (0.28–1.28)			12,556	7		0.0006
1:100 Rep8	0.86 (0.39–1.61)			10,979	8		0.0007
1:100 Rep9	1.45 (0.81–2.35)			11,371	14		0.0012
1:100 Rep10	0.94 (0.47–1.66)	**0.86 (0.10)**	37.5	12,508	10	8.6 (2.7)	0.0008
1:1000 Rep1	0.18 (0.03–0.57)			13,182	2		0.0002
1:1000 Rep2	0.27 (0.06–0.7)			13,343	3		0.0002
1:1000 Rep3	0.09 (0.004–0.44)			12,921	1		0.0001
1:1000 Rep4	0.29 (0.07–0.77)			12,112	3		0.0002
1:1000 Rep5	0.33 (0.10–0.77)			14,343	4		0.0003
1:1000 Rep6	0.0 (0.00–0.32)			11,181	0		0
1:1000 Rep7	0.31 (0.07–0.81)			11,573	3		0.0003
1:1000 Rep8	0.27 (0.066–0.73)			12,788	3		0.0002
1:1000 Rep9	0.18 (0.03–0.59)			12,891	2		0.0002
1:1000 Rep10	0.28 (0.07–0.74)	0.22 (0.03)	45.7	12,678	3	2.4 (0.76)	0.0002
NTC1	0 (0.0–0.28)			12,442	0		–
NTC2	0 (0.0–0.21)			16,933	0		–
NTC3	0 (0.0–0.25)			14,014	0		–

Note: Concentration for limit of detection is shown in bold. *Replicate 1 in the undiluted sample (in italic font) was discarded from the analysis because of low droplet count: hence, group means are calculated only with nine replicates.

no significant differences in the COX1 concentration between the droplet generation events were detected (Wilcoxon rank sum test: $W = 18$, $p = 1.000$), the median shift being 0.27 copies/μl (Figure 2).

Regression analysis showed that the concentration of the 10-fold dilution series had good linearity ($R^2 > .99$, $p < .001$) (Figure 4). The dynamic range of the ddPCR assay reached as low as 1:1000 dilution (mean concentration =0.22, SE =0.03 copies/μl) (Table 4). However, one replicate did not show any amplification; hence, LOD was determined to be 0.86 COX1 copies/μl (1:100 dilution), as all 10 replicates had more than 3 positive droplets (Table 4). %CV was lowest for the undiluted original DNA sample and highest in 1:1000 dilution (Table 4).

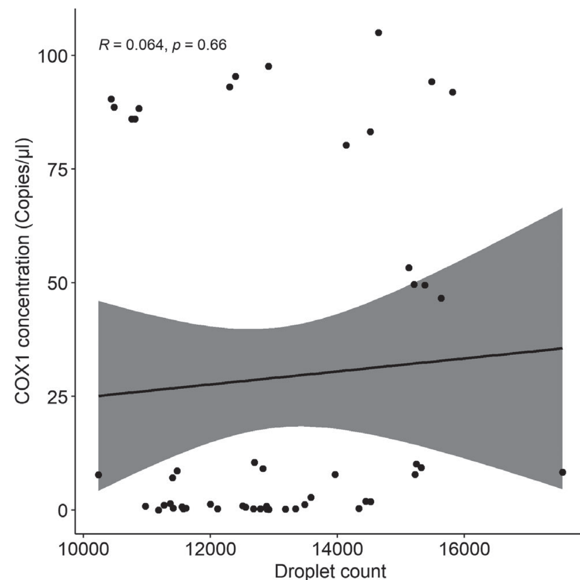

FIGURE 3 Non-significant Pearson's correlation between the COX1 concentration (copies/μl) and the droplet count ($n = 51$)

Absolute concentration measurements were independent of the droplet count (Pearson's correlation: $t = 0.449$, df =49, $p = .655$) (Figure 3). The droplet count varied from 10,239 to 17,553. Positive and negative droplets were easily separated from each other with threshold set to amplitude 13,033, mean amplitude for positives being 16,788, and negatives 5028. Some "rain" between the positive and negative clusters was observed, potentially because of the formation of primer dimer and the presence of background amplification (Figures 2a and 4a). The ddPCR assay did not amplify other apicomplexans (*S. pygospionis* and *P. pygospionis*) possibly present in the total DNA extractions from hosts (concentration 0 copies/μl) (Figure 2a).

3.2 | Prevalence of infection within host populations

Prevalence of infection differed between the populations ($X^2 = 64.9$, df =3, $p > .001$). The highest proportion of infected hosts ranged from 85% to 100% in Herslev, where it was 94.9% more likely that the worms were infected than in Lammefjord, 77.1% more likely than in Vellerup, and 91.8% than in Lynæs (Table 5). The lowest prevalence was found in Lammefjord, where fewer than 50% of hosts were infected throughout the sampling period (Figure 4, Table 5). No clear seasonal pattern common to the four populations was found ($X^2 = 4.3$, df =4, $p = .36$). The highest prevalence was observed in

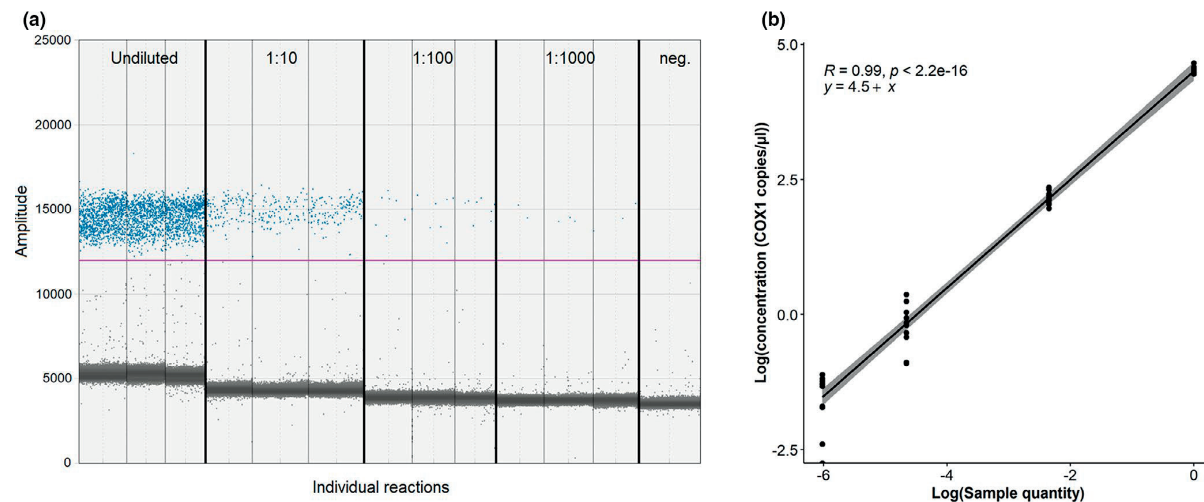

FIGURE 4 Ten-fold dilution series to detect the lowest limit for agamococcidian detection for the ddPCR assay. (a) 1D ddPCR plot. *x*-axis shows an example of individual reactions for the undiluted, 1:10, 1:100, and 1:1000 diluted replicates, and *y*-axis shows the amplitude of the fluorescence. Positive reactions (blue) and negative reactions (grey) are separated by manually set threshold (pink line, at amplitude 11,309). Negative controls (neg) did not show any amplification above the threshold. (b) Dilution series sample quantity in log scale plotted against COX1 concentration (copies/μl) in log scale in each replicate. Significant correlation coefficient ($r^2 = .99$, $p < .001$) between the absolute concentration (COX1 copies/μl) and 10-fold dilution series shows good linearity of the assay

TABLE 5 Pooled logistic regression odds ratios for prevalence of infection

		Odds ratio (95% CI)	SE	*t*	df	*p*-value
	(Intercept)	6.231 (1.83–21.26)	0.623	2.899	487	<.005
Population	Lammefjord	0.051 (0.02–0.11)	0.381	−7.822	487	<.001
	Vellerup	0.229 (0.11–0.50)	0.393	−3.750	487	<.001
	Lynæs	0.082 (0.04–0.18)	0.400	−6.242	487	<.001
Month	May	1.958 (0.78–4.93)	0.470	1.433	487	.152
	August	1.025 (0.54–1.96)	0.331	0.071	487	.944
	October	1.135 (0.59–2.20)	0.338	0.375	487	.708
	November	1.799 (0.87–3.73)	0.371	1.588	487	.113
Length		1.001 (1.0001–1.001)	0.000	2.199	487	.028
Density		0.999 (0.9994–1.000)	0.000	−3.912	487	<.001

Note: The distribution error function is binomial with a logistic link function. The references for population and month were Herslev and March, respectively. Altogether 499 samples were utilized in this model, including 19–28 individuals per population in each month.

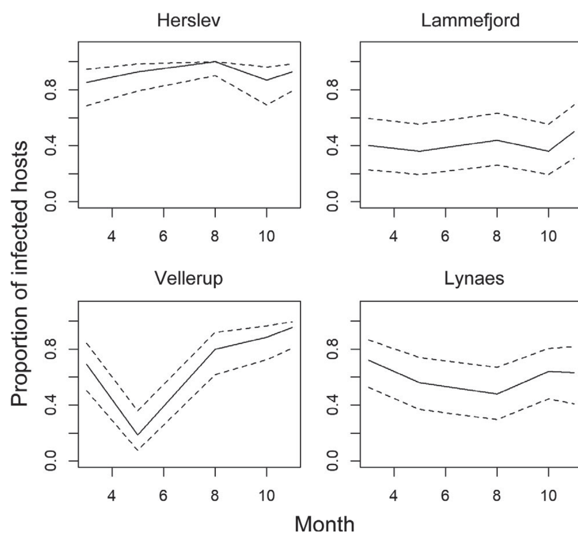

FIGURE 5 Proportion of infected host individuals through the sampling period in each population. Dashed lines indicate the 95% confidence intervals

Herslev in August (of all studied populations and sampling times) and the lowest in Vellerup in May (Figure 5). Although the probability of being infected increased towards November, the increase was not significant (Table 5).

Larger worms had an increased probability to be infected, but the effect was small: For every micrometer increase in length, the probability of being infected was 0.1% higher (Table 5). When host population densities were high, the prevalence of infection was significantly lower, but the effect was small (Table 5). For every host individual increase within a square meter, the probability of infection decreased to 0.04%. Organic content (%) in the sediment was found to decrease the proportion of infected hosts by 67%, but the effect was not significant ($z = -0.994$, df =20.1, $p = .332$), and the predictor was dropped from the final model (likelihood-ratio test D3: $p = .335$).

We obtained similar results when the analysis was restricted to only complete cases (excluding samples from October). However, logistic regression with multiple imputation was generally more efficient as can be seen from the smaller confidence intervals and lower *p*-values (Table 6). Also, infection load peaked in Lammefjord and Lynæs in October, which would not have been observed in the complete cases model.

3.3 | Infection load in natural host populations

The largest range of agamococcidian COX1 gene copies detected by ddPCR was in the Herslev population, ranging between 0.86 and 1700.98 copies/µl. In Lammefjord, the range of detection was from 0.86 to 53.70 copies/µl, in Vellerup from 0.86 to 807.62 copies/µl, and in Lynæs from 0.86 to 665.30 copies/µl.

The agamococcidian infection was highly aggregated in most populations and throughout the sampling period (Table 7.) Variance to mean ratio was high in all populations except in Lammefjord in May and in August, when also the mean infection load was lowest (from 0.4 to 1.62 COX1 copies/ng of total DNA). The infection was most aggregated in October (Lammefjord and Lynæs) and in November (Herslev and Vellerup).

Linear regression analysis ($r^2 = .272$) showed that the infection load differed between populations ($F = 29.30$, df =3, $p > .001$). In Herslev, the infection load was high throughout the sampling season, ranging from 101.10 to 349.20 copies/ng total DNA. The lowest infection load was found in Lammefjord, where the range was from 0.40 to 72.20 copies/ng total DNA. The infection load changed seasonally ($F = 8.08$, df =4, $p > .001$) being highest in October (Figure 6, Table 8). Overall, the highest mean infection load was found in Vellerup ($\bar{x}$ = 351.6 copies/ng of total DNA, SE =126) in August, and the lowest in Lammefjord in March ($\bar{x}$ = 0.4 copies/ng of total DNA, SE =0.1) (Table 7, Figure 6).

Higher organic content in the sediment was associated with lower infection load, but the effect was not significant ($t = -1.579$, df =140, $p = .117$). Also, the length of the host did not have a significant effect

TABLE 6 Logistic regression with complete cases only (missing data from October)

		Estimate (95% CI)	SE	*z*-value	df	*p*-value
	(Intercept)	1.764 (0.38 to 3.29)	0.718	2.457	397	.014
Population	Lammefjord	−3.014 (−3.94 to −2.20)	0.442	−6.826	397	<.001
	Vellerup	−1.766 (−2.70 to −0.92)	0.449	−3.929	397	<.001
	Lynæs	−2.767 (−3.74 to −1.89)	0.467	−5.918	397	<.001
Month	May	0.708 (−0.23 to 1.68)	0.485	1.458	397	.145
	August	0.020 (−0.63 to 0.67)	0.332	0.060	397	.952
	November	0.601 (−0.13 to 1.35)	0.376	1.597	397	.110
Length		0.001 (0.000 to 0.001)	0.000	2.303	397	.021
Density		−0.0003 (−0.001 to 0.000)	0.000	−3.790	397	<.001

Note: The distribution error function is binomial with a logistic link function. The references for population and month are Herslev and March, respectively. Altogether 398 samples were utilized in this model, including 19–28 individuals per population in each month.

TABLE 7 The prevalence of infection (%) and number of infected hosts (*n*), mean infection load (COX1 copies/ng of total DNA) ($\bar{x}$), the standard error for the mean (SE), variance (s^2), and variance to mean ratio ($s^2/\bar{x}$) in the studied populations

		% (*n*)	$\bar{x}$	SE	s^2	$s^2/\bar{x}$
March	Herslev	85.2 (23)	101.10	46.10	48,915.70	483.83
	Lammefjord	40.0 (10)	0.40	0.10	0.10	0.30
	Vellerup	69.2 (18)	1.30	0.50	3.70	2.80
	Lynaes	72.0 (18)	18.20	13.60	3333.00	183.10
May	Herslev	92.9 (26)	104.90	43.50	49,265.50	469.60
	Lammefjord	36.0 (9)	1.62	0.50	2.16	1.30
	Vellerup	18.5 (5)	59.70	58.20	16,937.52	283.71
	Lynaes	56.0 (14)	1.10	0.30	1.50	1.38
August	Herslev	100.0 (24)	150.45	64.30	99,181.60	659.20
	Lammefjord	44.0 (11)	0.75	0.20	0.44	0.59
	Vellerup	80.0 (20)	351.60	126.00	317,762.80	903.76
	Lynaes	48.0 (12)	2.61	1.41	24.01	17.03
October	Herslev	87.0 (20)	210.20	106.60	227,294.40	1081.30
	Lammefjord	36.0 (9)	72.20	65.20	38,222.87	529.40
	Vellerup	88.5 (23)	140.40	8.40	14,8597.40	1058.39
	Lynaes	64.0 (16)	250.40	197.60	62,4783.70	2495.14
November	Herslev	92.9 (26)	349.20	145.20	548,069.20	1569.50
	Lammefjord	50.0 (12)	24.40	23.90	6826.50	279.78
	Vellerup	95.5 (21)	164.50	10.10	227,784.40	1384.71
	Lynaes	63.2 (12)	19.00	15.60	2933.20	154.38

on the infection load ($t = -0.495$, df =315, $p = .621$), nor did the host population density ($t = 0.806$, df =316, $p = .421$), and these variables were left out of the final model, which was performed for the original, non-pooled data without multiple imputation.

4 | DISCUSSION

In this study, we show that ddPCR can be used as a reliable tool to quantify symbionts from whole host DNA extracts. We developed a ddPCR assay to detect and quantify an undescribed agamococcidian based on its COX1 gene, and we demonstrated its use in studying infection dynamics by documenting the prevalence of the symbiont and the infection load in four seasonally sampled populations of its host, *P. elegans*. ddPCR can enable further investigation of cryptic symbiotic interactions that are difficult to carry out with other molecular methods and impossible with morphological methods alone. Here, the agamococcidian COX1 was detected in amounts ranging from 0.04 to >3000 copies per ng of total DNA extracted from hosts (mixtures of host and symbiont DNA).

As expected for ddPCR, our assay was reproducible and precise, similar to ddPCR assays used for symbiont quantification in

previous studies (Koepfli et al., 2016; Srisutham et al., 2017; Wilson et al., 2015). Results were not affected by droplet generation event (Figure 2), and variation in the concentration was small especially when the number of copies/μl was high (Figures 2 and 3). The precision of ddPCR is expected to increase with an increasing number of partitions (Quan et al., 2018). In our experiments, the droplet count varied from 10,239 to 17,553, which is typical for droplet-based methods, and it did not correlate with target concentration (Figure 3). Additionally, the precision is affected by the average number of target molecules per droplet (λ), since an uneven distribution of template across the partitions might cause a decrease in precision for very low target concentration samples (Hindson et al., 2013; Strain et al., 2013). Our experiments showed an even distribution of targets per droplet; however, low λ and higher %CV values were detected for very low target concentrations (Tables 3 and 4).

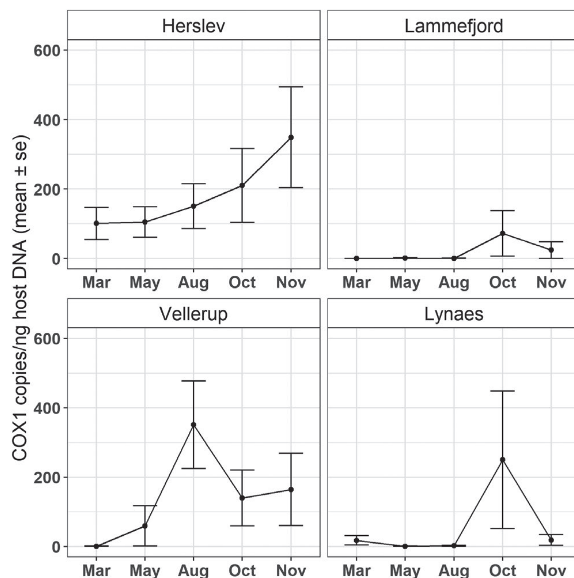

FIGURE 6 Mean infection load (COX1 copies/ng total DNA) in the four populations over the sampling period. Error bars indicate the standard error of the mean

As ddPCR results in absolute concentration and no standard curves are needed (Hindson et al., 2013), the method is particularly applicable for quantifying undescribed symbionts from total DNA extracts of hosts, when extraction of the symbionts is not feasible to provide the reference material needed when preparing standard curves (Salipante & Jerome, 2019). Importantly, our assay can detect symbionts at a very low density (0.86 copies/μl), which might realistically be expected for agamococcidians and other apicomplexans. Calculation of infection loads for agamococcidians has not been attempted previously, but Miroliubova et al. (2020) remarked that they found from a few up to several hundreds of individual rhytidocystids within the polychaete *Ophelia limacine*. Moreover, the assay developed here did not amplify the other apicomplexan symbionts (*S. pygospionis* and *P. pygospionis*) that might also be present within the hosts, showing the specificity of the designed primers. Hence, the ddPCR assay not only enables monitoring of symbionts that are otherwise difficult to quantify but also makes it possible to study co-infections of multiple apicomplexan species across host populations efficiently in terms of both time and labor (A.-L. Hiillos et al., in prep).

However, because our assay is based on the mitochondrial COX1 gene, the quantification of gene copies given by the ddPCR assay does not indicate the exact number of symbiont cells that are present in a single host individual or how many individuals is the minimal level of detection. A single symbiont cell can potentially harbor multiple copies of the mitochondrial marker. For example, in *S. pygospionis*, the number of mitochondria increases with cell size (Paskerova et al., 2018). To achieve more accurate estimates of the symbiont count, a single copy nuclear marker would be better suited for the assay. Considering how well the target symbiont is known and whether genetic data from the symbiont is available, it could be difficult to design primers with sufficient specificity to use with total host DNA extracts because of conservation of gene sequences shared with the host or other related symbiont species. We used the COXI gene fragment because it was available and sufficiently different from the host COXI sequence (Supporting information 1) and because the COXI gene has shown potential for DNA barcoding of some protists (Pawlowski et al., 2012) including apicomplexans (Ogedengbe et al., 2011). It would be interesting to follow up our

TABLE 8 Linear regression coefficients for the log-transformed infection load

		Estimate (95% CI)	SE	*t*-statistic	df	*p*-value
	(Intercept)	1.70 (1.05 to 2.35)	0.330	3.630	319	<.001
Population	Lammefjord	−3.16 (−3.92 to −2.41)	0.386	−8.20	319	<.001
	Vellerup	−0.99 (−1.38 to −0.32)	0.330	−2.99	319	<.005
	Lynæs	−2.28 (−2.96 to −1.61)	0.345	−6.61	319	<.001
Month	May	0.93 (0.10 to 1.76)	0.422	2.19	319	.029
	August	1.53 (0.75 to 2.31)	0.396	3.86	319	<.001
	October	1.93 (1.16 to 2.71)	0.394	4.90	319	<.001
	November	1.82 (1.05 to 2.59)	0.391	4.65	319	<.001

Note: The references for population and month were Herslev and March, respectively. The distribution error function is normal. Because only infected hosts were used in the analysis, the sample size was 329 in total, including 5–26 individuals per population in each month. Analysis was performed without multiple imputation since the variables with missing data (length and organic content %) were not significant.

study using a different assay that could provide more exact counts. Regardless, our ddPCR assay based on COXI not only allows for detection of the undescribed agamococcidian but also provides an indication of the symbiont infection load, allowing for further insight into symbiont dynamics and biological interactions between symbiont and host.

Use of our ddPCR assay to survey natural populations of the host revealed a dynamic pattern of infection by the agamococcidian in the studied host populations. The proportion of infected *P. elegans* was high overall (85.7%), but it differed between the populations, and it remained constant throughout the sampling season in most populations. The highest prevalence was found in Herslev, where almost all hosts were infected. Prevalence was low in Lammefjord, ranging from 36% to 50%, and intermediate in Lynaes, where it ranged from 48% to 72%. Only in Vellerup, prevalence changed over the season, from high (69.2%) in March to very low in May (18.5%), and increasing again towards fall so that 95% of hosts were infected in November. Similarly, the highest mean infection load was found in Herslev and the lowest in Lammefjord. Infection load changed seasonally increasing towards fall: peaking earliest in August in Vellerup, but otherwise highest in October (Lammefjord and Lynaes) and November (Herslev). Furthermore, infection load was highly aggregated in all populations, meaning that few individuals in the population harbor majority of the symbionts. The symbiont distribution within infected hosts also showed seasonal dynamics, with low or almost even variance to mean ratios in some populations in spring and summer but more aggregation in the fall. Although apicomplexan dynamics in marine environments have not been extensively studied, seasonal changes in prevalence and/or infection intensity have been observed in some host–symbiont systems. For example, apicomplexan infection in the Iceland scallop showed high prevalence throughout the seasons, with infection load being highest in spring (Kristmundsson et al., 2015). In another study, prevalence of apicomplexans infecting reef-building corals was low during summer months and increased towards fall (Kirk et al., 2013). In contrast, some marine apicomplexan infections are found to remain stable throughout seasons (Halliday-Isaac et al., 2021), suggesting that the infection dynamics of marine apicomplexans are case specific and require more thorough examination.

Apicomplexans are generally expected to be parasitic (Morrison, 2009), but how the agamococcidian studied here affects the host's fitness is not known. Aggregated distribution within hosts is typical for parasitic symbionts (Anderson & May, 1978), and we expect that the same factors influencing parasite dynamics on a general level, specifically encounter rates and host susceptibility (Schmid-Hempel, 2011), could also be relevant for the studied species. Marine apicomplexans are thought to have simple life cycles with one host species and passive oral–fecal transmission among hosts (e.g., Leander, 2007). Therefore, high host densities are expected to increase prevalence and infection loads (Anderson & May, 1978; Arneberg et al., 1998) since symbiont transmission is more efficient because of increased encounter rates (Patterson & Ruckstuhl, 2013; Rifkin et al., 2012). However, we found that when host density was highest (in May), prevalence of infection did not increase accordingly. In addition, the infection load was lower when host density was high, although the estimate was not significant. A similar non-significant negative effect of host density on prevalence was detected in apicomplexans infecting amphipods (Grunberg & Sukhdeo, 2017). Those results, together with ours, suggest that the relationship between host density and prevalence or infection load is not necessarily straightforward and is possibly modified by other characteristics of both host and symbiont biology.

For instance, seasonality in host reproduction and reproduction strategy might affect prevalence and infection loads (Šimková et al., 2005; White et al., 1996) via changes in encounter rates and susceptibility. The studied host populations show seasonal dynamics in their reproduction, with reproductive peaks occurring in both spring and fall (Thonig et al., 2016). We observed that infection load and symbiont aggregation were high in October and November when the proportion of reproducing hosts also increases, perhaps because of an increase in food consumption to enable gamete production and consequential exposure to agamococcidian oocysts. However, we did not observe a similar peak in spring. Additionally, the host populations differ in the main mode for larval development (Thonig et al., 2016). Prevalence and infection loads were highest throughout the sampling season in Herslev, where the worms are known to produce mainly large, non-dispersing benthic larvae. Interestingly, low prevalence and infection loads were detected in Lammefjord and Lynæs, where the main developmental mode is via planktonic larvae, which are capable of dispersing. Because of these developmental differences, a more constant supply of susceptible hosts might be present in Herslev compared with Lammefjord and Lynæs, where influx of new cohorts via planktonic larvae might be less predictable. The pattern and association of infection with developmental mode demand additional study.

Another host characteristic that affects symbiont encounter is host body size (e.g., Taskinen & Valtonen, 1995). Larger size is generally connected to older age or increased consumption rates, which could further increase encounter rates with the symbiont. We found that larger worms were more likely to be infected, but host size did not affect the infection load. In a system where two apicomplexans are infecting the same host species, Grunberg and Sukhdeo (2017) found that larger and older hosts had higher infection load of one apicomplexan species but not the other, leading them to propose that changes in host feeding patterns along with seasonal changes in host demographics might drive changes in symbiont abundance. The host species in our study, *P. elegans*, can both filter feed and deposit feed (Anger et al., 1986), deposit feeding being more common in larger worms (Hentschel, 1998), which could explain our observation of larger worms being more likely to be infected. As the available food type can affect the grazing behavior in polychaetes (Jordana et al., 2001), filter feeding in *P. elegans* might be more common in spring when phytoplankton is more abundant. A change in feeding behavior with season might reflect the infection load patterns observed in this study, since hosts are more likely to encounter symbiont infective stages while deposit feeding. This could also

explain why we did not observe an increase in parasite load during the spring reproductive peak. Even though reproducing hosts have higher consumption rates to support developing gametes, if the increased consumption is through filter feeding in spring, agamococcidian oocysts could be avoided. Furthermore, the symbiont's life history might also affect the encounter rates. Many marine symbionts accelerate their reproduction rate inside their hosts during warm seasons (Overstreet & Lotz, 2016). Consequently, there could be a progressive accumulation of oocysts in the sediment towards fall. Since apicomplexan oocysts are known to be very persistent (Clopton et al., 2016), oocysts produced in summer can remain viable until fall when hosts encounter rate possibly is higher because of the afore-mentioned features in host biology.

We observed differences among host populations and did not find a common seasonal pattern of infection, suggesting that environmental factors unrelated to broad seasonal changes could be influencing symbiont and host interactions. In this study, the environmental factors are all represented by the organic content % in the sediment, since other variables measured (salinity, temperature, and sediment characteristics) were correlated with host density. We chose to retain organic content in our analysis since it measures the amount of nutrients available for the host to use (e.g., Cheng et al., 1993). Higher organic content was found to lower infection load and the prevalence of infection, even though the effect was not statistically significant. Low organic content in sediment has been correlated with larger foraging area in the polychaete *Streblospio benedicti* (Kihslinger & Woodin, 2000) and could therefore increase the encounter rate of the host with symbiont oocysts and lead to heavier infection. The pattern could also indicate that hosts living in a habitat with higher organic content could be in a better condition and thus have better immunity against the infection. However, as the nature of the interaction between the symbiont and its host (parasitic or mutualistic) and possible immunity is not currently known, further studies are needed. In addition, it is important to remember that one of the other environmental variables correlated with organic content % might be more relevant biologically for explaining the infection patterns, and other unmeasured environmental variables cannot be discounted.

5 | CONCLUSION

We have shown that ddPCR can be used effectively to detect symbionts and quantify infections from DNA extracts of hosts despite challenges of working with cryptic and undescribed symbiont species. Our assay reliably targets an agamococcidian symbiont in a marine polychaete, *P. elegans*, and provides specific quantification of infection load despite the fact that DNA pools could also contain DNA from other, related apicomplexan species as well as host DNA. We surveyed four natural host populations sampled seasonally and found that infection was population specific and dynamic. A common seasonal pattern of prevalence was not detected. Since very few studies have been able to survey agamococcidians or other marine apicomplexans, our results suggest a concrete avenue towards better understanding of the interaction of marine apicomplexans and their hosts. Effective and efficient tools, such as the ddPCR assay introduced in this study, are required for monitoring the host–symbiont dynamics and the consequences of their biological interactions.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Anna-Lotta Hiillos: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Validation (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal). **Anne Thonig:** Data curation (equal); Investigation (equal); Methodology (equal); Resources (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Karelyn Emily Knott:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (lead); Project administration (equal); Resources (equal); Supervision (lead); Validation (equal); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

All data and R scripts are uploaded to Dryad repository (https://doi.org/10.5061/dryad.jwstqjq90). Sequence data generated in this study from two isolates of agamococcidian COX1 with accession numbers OK562425 and OK562426 will be available in NCBI GenBank upon acceptance.

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II

COINFECTION PATTERNS OF TWO MARINE APICOMPLEXANS ARE NOT ASSOCIATED WITH GENETIC DIVERSITY OF THEIR POLYCHAETE HOST

by

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ORIGINAL ARTICLE

Coinfection patterns of two marine apicomplexans are not associated with genetic diversity of their polychaete host

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Abstract

Coinfections of two or more parasites within one host are more of a rule than an exception in nature. Interactions between coinfecting parasites can greatly affect their abundance and prevalence. Characteristics of the host, such as genetic diversity, can also affect the infection dynamics of coinfecting parasites. Here, we investigate for the first time the association of coinfection patterns of two marine apicomplexans, *Rhytidocystis* sp. and *Selenidium pygospionis*, with the genetic diversity of their host, the polychaete *Pygospio elegans*, from natural populations. Host genetic diversity was determined with seven microsatellite loci and summarized as allelic richness, inbreeding coefficient, and individual heterozygosity. We detected nonsignificant correlations between infection loads and both individual host heterozygosity and population genetic diversity. Prevalence and infection load of *Rhytidocystis* sp. were higher than those of *S. pygospionis,* and both varied spatially. Coinfections were common, and almost all hosts infected by *S. pygospionis* were also infected by *Rhytidocystis* sp. *Rhytidocystis* sp. infection load was significantly higher in dual infections. Our results suggest that factors other than host genetic diversity might be more important in marine apicomplexan infection patterns and experimental approaches would be needed to further determine how interactions between the apicomplexans and their host influence infection.

KEYWORDS

gregarines, heterozygosity, host–symbiont interactions, Marosporida, symbiont–symbiont interactions

AS more than half of all living organisms are parasites (de Meeûs & Renaud, 2002), most infections in nature consist of multiple parasite strains or species (de Meeûs & Renaud, 2002; Petney & Andrews, 1998). Interactions among coinfecting parasites can strongly influence parasite dynamics (both within and among hosts) and host populations (Clerc et al., 2019; Rovenholt & Tate, 2022; Seabloom et al., 2015), as coinfections can lead to competitive exclusion of other parasites (Dib et al., 2008; Dobson, 1985; Read & Taylor, 2001), mutualistic coexistence (Jaenike et al., 2010), or facilitation, where one parasite provides suitable conditions for infection of the other (Behnke et al., 2009; Zélé et al., 2014; Zélé et al., 2018). From the host perspective, the factors

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affecting coinfection patterns (e.g. environment, host behavior, or genetics) can be complex to disentangle (Viney & Graham, 2013), and the infection outcome can be difficult to predict (Pedersen & Fenton, 2007; Petney & Andrews, 1998), but coinfections can increase disease severity (Alizon et al., 2013; Gibson et al., 2011; Manzi et al., 2021), host susceptibility to other infections (Cattadori et al., 2007), and the overall degree of parasite epidemics (Susi et al., 2015).

Another important factor in host–parasite interactions is host genetic diversity (Bérénos et al., 2011; Ekroth et al., 2019) as it provides resilience against infections and allows hosts to coevolve with their parasites (Webster & Woolhouse, 1999). Parasite-induced selection, on the contrary, can affect the genetic diversity of host populations (Haldane, 1949). For example, the presence of parasites maintains genetic diversity in small populations by removing less heterozygous hosts (Coltman et al., 1999) and can reduce population-level inbreeding (Cabalzar et al., 2019; Kaunisto et al., 2013). According to diversity-disease hypothesis, decreased genetic diversity in host populations increases the occurrence of infection (Elton, 1958; Garrett & Mundt, 1999; Keesing et al., 2006). In contrast, high host population genetic diversity reduces the likelihood that a parasite encounters a susceptible host due to the increased chance that an individual is resistant to infection (Anderson & May, 1982; Keesing et al., 2006) and can limit parasite spread (Ostfeld & Keesing, 2012). Multiple empirical observations and experiments have given support to the hypothesis. For instance, Altermatt and Ebert (2008) found that spread of a microsporidian parasite is significantly more efficient in *Daphnia magna* populations of low genetic diversity, compared to a population with high diversity. At the individual level, low genetic variation has been connected to increased susceptibility to infections (Kaunisto et al., 2013; Whitehorn et al., 2011) and high genetic diversity has been shown to increase individual host's resistance to infection (Isomursu et al., 2012; King & Lively, 2012).

Apicomplexans are a diverse group of microbial eukaryotes infecting a wide variety of hosts in terrestrial and aquatic environments (Seeber & Steinfelder, 2016). The group is described as obligately parasitic and includes some of the most notorious and well-studied parasites (e.g. the causative agent of malaria, *Plasmodium*) (Morrison, 2009). However, for many apicomplexans, especially the marine species, the nature of their interaction with their hosts is not currently known. For instance, gregarines are known to be common and prevalent symbionts of invertebrates, but their interactions with their hosts can range from mutualistic to parasitic associations (Rueckert et al., 2019). Research on marine apicomplexan infections in relation to host genetic diversity has also been scarce, and studying if such associations exist might help resolve the nature of the interaction. In some invertebrates, apicomplexans have higher infection loads within hosts with low heterozygosity (Kaunisto et al., 2013), while in others, no association has been found (Velavan et al., 2009). Coinfections of marine gregarines have been reported (Paskerova et al., 2018, 2021), but whether their infection is related to host genetic diversity has not been studied before.

In this study, we examined marine apicomplexan infections, coinfections, and their associations with host genetic diversity in three natural populations of the polychaete worm, *Pygospio elegans*. We determined genetic diversity by genotyping individual hosts at seven neutrally evolving microsatellite loci (Thonig et al., 2017) and inspected infection patterns of an undescribed marosporidian, *Rhytidocystis* sp., and the gregarine species, *Selenidium pygospionis*, that live in symbiosis with the host. Our aim was to determine whether infection dynamics are associated with differences in population genetic diversity and heterozygosity among individual hosts. Heterozygosity at microsatellite loci can be correlated positively with fitness, such as fecundity (Amos et al., 2001; Charpentier et al., 2005), and lifetime reproductive success (Slate et al., 2000), and multiple studies have used heterozygosity–fitness correlation approach in studying the effect of host genetic diversity on parasite infection (Acevedo-Whitehouse et al., 2003; Coltman et al., 1999; Portanier et al., 2019). As we do not know whether the studied apicomplexan species cause harm to their host, they are referred to here as symbionts (Rueckert et al., 2019). We still expect that higher population genetic diversity would be associated with lower infection loads and that genetically more diverse hosts (with higher heterozygosity) are less susceptible to infection and exhibit lower apicomplexan prevalence and infection loads.

MATERIALS AND METHODS

Study species

The host, *Pygospio elegans* Claparède, 1863 is a small tube-dwelling marine polychaete worm that has a circumboreal distribution in sandy coastal habitats throughout the Northern Hemisphere. It has an important role in benthic communities, as it can reach high densities (Bolam, 2004; Bolam & Fernandes, 2003) and is an important prey animal to other invertebrates and fish (Mattila, 1997). At least three protist species in the phylum Apicomplexa are known to infect *P. elegans*: an archigregarine, *Selenidium pygospionis* (Paskerova et al., 2018); an eugregarine *Polyrhabdina pygospionis* (Paskerova et al., 2021); and an undescribed marosporidian (Class Marosporida; Mathur et al., 2020) in the genus Rhytidocystis (Hiillos et al., 2021), from now on referred to as *Rhytidocystis* sp. All of these apicomplexans infect the worm's intestine.

Sample collection

Host samples were collected from three populations, Cramond Beach (55°58′N, 3°17′53 W, Edinburgh, UK), Herslev (55°40′N, 11°59′E, DK), and Vellerup (55°44′N 11°52′E, DK). These populations were chosen because they have been studied extensively previously (Bolam, 2004; Hiillos et al., 2021; Thonig et al., 2016, 2017). All samples were collected in early November in consecutive years 2018 and 2019. Sand tubes containing *P. elegans* were collected from the top layer of sediment (below water in nontidal Herslev and Vellerup, or from tidal pools during the low tide in Cramond Beach) using a 1-mm-mesh sieve. Twenty to forty live worms from each site were separated from their tubes under a dissecting microscope, stored individually in DNA/RNA Shield (Zymo Research) on site, and transported to the University of Jyväskylä.

DNA extraction and microsatellite genotyping

DNA was extracted from complete specimens using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, and DNA concentration was measured with the Qubit 4.0 Fluorometer with 1× dsDNA HS Assay (Thermo Fisher Scientific). Subsequently, seven microsatellite loci were amplified using 1× Qiagen Multiplex PCR Master Mix. Reaction volume was 10 µl, with each primer in 0.2 µmol/L and 1 µl DNA. The loci were grouped into two multiplex panels: Multiplex 1 contained loci Pe307, Pe385, and Pe6; Multiplex 2 contained loci Pe19, Pe234, Pe294, and Pe369. Loci Pe307, Pe385, Pe234, Pe294, and Pe369 were originally described in Thonig et al., 2017, and loci Pe6 and Pe19 were originally described in Kesäniemi et al., 2012 (Table S1). These markers have been used successfully to assess population genetic structure in multiple *P. elegans* populations previously (Kesäniemi et al., 2012, 2014; Thonig et al., 2017). PCR conditions were as follows: initial activation step of 15 min at 95°C followed by 30 cycles of 30 s at 95°C, 90 s at 60°C, and 60 s at 72°C, and a final extension for 30 min at 60°C. The resulting fragments were separated on an ABI PRISM 3130xl and analyzed with GeneMapper® v.5 Software (Applied Biosystems). Automated allele sizing in GeneMapper was checked manually for each sample, and alleles that occurred only once in the dataset were double-checked and confirmed in the raw data. Hosts that had missing information for more than two loci were discarded from further analysis, leaving 18–36 individuals per sample.

Population genetic diversity and individual heterozygosity

For each sample, observed heterozygosity and expected heterozygosity (H_O and H_E) averaged over all loci were calculated using GenAlEx v. 6.501 (Peakall & Smouse, 2012) plug-in in Microsoft Excel (2016). Genetic variability within different populations was estimated as mean allelic richness (AR) calculated with the rarefaction method using the smallest sample size. Inbreeding coefficient (F_{IS}) indicating nonrandom mating within sampling sites, and pairwise differentiation among sites (F_{ST}) were estimated with Weir and Cockerham (1984) estimators of F-statistics. AR, F_{IS}, and pairwise F_{ST} values were calculated in FSTAT v. 2.9.4 (Goudet, 2003; updated from Goudet, 1995). Statistical testing for F_{IS} was done using 499 permutations with a significance level of $\alpha = 0.05$, and statistical significance of pairwise F_{ST} values ($\alpha = 0.05$) was obtained with a Bonferroni correction after 60 permutations. Comparison of the estimated population parameters (H_O, H_E, AR, F_{IS}) between populations and years was done with permutation tests in FSTAT with 10,000 permutations. Analysis of molecular variance (AMOVA) among populations for the seven loci was assessed with GenAlEx. Test of significance was performed using 9999 permutations within the total dataset. Individual heterozygosity was estimated for each host by calculating the proportion of heterozygous loci out of the total number of loci genotyped, taking into account missing data (5–7 loci per individual) (Coltman et al., 1999).

Apicomplexan detection and quantification

The prevalence of *Rhytidocystis* sp. and *S. pygospionis*, as well as their infection loads, was assessed with droplet digital PCR (ddPCR) using Bio-Rad's QX200™ Droplet Digital™ PCR System. ddPCR is based on partitioning and randomly distributing the sample into nanoliter-sized droplets before PCR amplification, which takes place in each droplet separately. After amplification, the fraction of positive droplets is determined and the concentration of the target can be estimated (Hindson et al., 2011, 2013). As ddPCR gives absolute quantification and no standard curves are needed, it is particularly useful in quantifying symbionts that have little genetic information available and are unculturable outside their hosts (Hiillos et al., 2021).

In our detection and quantification assays, we used primers targeting the mitochondrial *cox1* gene of *Rhytidocystis* sp. and *S. pygospionis* (Table S2) as they showed sufficient divergence from the host's *cox1* sequence and from each other. The *cox1* gene has been used previously in species barcoding studies of protists (Pawlowski et al., 2012). The *cox1* gene is also currently the only available genetic marker for *Rhytidocystis* sp. (Hiillos et al., 2021). Additionally, because another *P. elegans*-infecting gregarine (*Polyrhabdina pygospionis*) was not studied here, the use of *cox1* ensured the assays were specific, as *P. pygospionis* has lost *cox1* from its mitochondrial genome (Mathur et al., 2021; Salomäki

et al., 2021). We used 2X QX200™ ddPCR™ EvaGreen® (Bio-Rad) reagent mix to prepare a 20 µl reaction mix per sample. Primers were added to the mix in 1 µM together with 4.6 µl of sterile water and 2 µl of DNA sample so that the final volume was 22 µl. Samples were partitioned into droplets with the QX200 Droplet Generator (Bio-Rad) using single-use DG8 cartridges, and the emulsion was made with 70 µl of Droplet Generation Oil (Bio-Rad) per sample. The resulting droplets were manually transferred with a multichannel pipet to a ddPCR™ 96-well PCR plate (Bio-Rad), which was heat-sealed with a foil cover. PCR conditions were as follows: initial denaturation at 95°C for 3 min, after which the denaturation, primer annealing, and target extension steps were repeated for 40 cycles, with a ramp rate of 2°C per second in each step. The denaturation step was done at 95°C for 30 s, annealing temperature for the primers was optimized at 58°C (*Rhytidocystis* sp. *cox1*) or 60°C (*S. pygospionis cox1*) for 1 min, and the target extension step was done at 72°C for 2 min. After the cycles, a signal stabilization step from 5 min at 4°C to 5 min at 90°C was added. Following the amplification, the droplets were immediately read with Bio-Rad's Droplet Reader. Data were analyzed with default ABS settings in QuantaSoft Analysis Pro 2.0 software (Bio-Rad). The ABS experiment estimates the concentration of the target in copies/µl of the final 1× ddPCR. Therefore, the infection load (copies/ng total DNA) for each sample was calculated as follows:

$$C_{ng} = \left(\frac{C_{ddPCR} \times V_r(\mu l)}{V_s(\mu l)} \right) / C_{DNA}$$

where C_{ng} is the number of copies/ng of total DNA, C_{ddPCR} is the reaction concentration (copies/µl) given by QuantaSoft Analysis Pro, V_r is the reaction mix volume, V_s is the sample volume, and C_{DNA} is the concentration (ng/µl) of total DNA. DNA from a host known to be highly infected with *Rhytidocystis* sp. and DNA isolated from individual cells of *S. pygospionis* were used as positive controls in the assay. Only reactions that had ≥10,000 droplets were included in further analyses. A threshold to separate the target positive and negative droplets was manually set in relation to the negative control by visual inspection (Figure S1).

The prevalence of the apicomplexans was measured as the proportion of infected *P. elegans* individuals in the sample. An individual host was considered infected if more than 0 copies of the target gene per ng of total DNA was detected with the ddPCR assay. As only similar-sized polychaetes were used and our previous study did not show any correlation between host size and infection load of *Rhytidocystis* sp. (Hiillos et al., 2021), we estimated infection load here as the number of *Rhytidocystis* sp. or *S. pygospionis cox1* gene copies/ng of total DNA and analyzed it only with infected hosts. However, since the copy number of *cox1* is not currently known for either of the apicomplexans and because each symbiont can potentially have multiple copies of the *cox1* gene, our estimation of the infection load does not indicate the exact number of the symbionts and can only be considered as a relative measure. Aggregation of the infection was inspected by calculating variance-to-mean ratios for each sample.

Statistical methods

Population genetic parameters, inbreeding coefficient (F_{IS}) and average allelic richness (AR), were correlated with mean infection load of both apicomplexans in each sample by Pearson's correlation coefficient. Differences in the individual heterozygosity between the samples were tested with analysis of variance (ANOVA). The prevalence of the apicomplexan infection was analyzed separately by logistic regression using population, sampling year, and prevalence of the other apicomplexan as predictors. The best-fitting model was chosen according to the lowest Akaike information criterion (AIC) (Table S3). We used linear regression to test the relationship between the infection loads of both apicomplexans with population, sampling year, and infection load of the other species as predictors. The best-fitting model was chosen according to highest adjusted r^2 value and lowest residual standard error (Table S3). In the model, infection loads were log-transformed to fit the assumption of normality. Dual infection frequency differences in each site were tested with the chi-squared test of independence. Pearson's correlation coefficient was used to test whether the infection loads of the apicomplexans are correlated with each other and whether host individual heterozygosity was correlated with the infection loads. Three hosts had an exceptionally high *Rhytidocystis* sp. infection load (>8000 copies/ng of DNA), and one host had exceptionally high *S. pygospionis* infection load (>2000 copies/ng of DNA) and were considered as outliers in the analysis. When the analyses were performed without these outliers, the correlation between the infection loads became weaker and the direction of correlations changed between infection loads and individual host heterozygosity (File S1; Figures S2–S4); hence, the final correlation analyses were performed without the outliers. All statistical analyses were conducted in R v. 4.0.5 (2021/03/31) (R Core Team, 2021).

RESULTS

Genetic diversity

The genetic diversity of the samples is described in Table 1. Allelic richness was higher in Vellerup than in Cramond Beach and Herslev, but the difference was not significant (p = 0.07, 10,000 permutations). Lowest

diversity among the samples was observed in Vellerup in 2019, when the inbreeding coefficient (F_{IS}) was exceptionally high (36.5%). In all samples except in Herslev 2019, a deficiency of heterozygotes was observed, and F_{IS} estimates were positive, indicating significant deviations from the Hardy–Weinberg equilibrium. Pairwise F_{ST} values showed genetic differentiation between Cramond Beach and Herslev populations in both years (Table 2). However, genetic differentiation was nonsignificant between the Cramond Beach and Vellerup populations in both 2018 and 2019, and Herslev and Vellerup in 2019 (Table 2). Analysis of molecular variance (AMOVA) showed that the majority of variation was found within individuals (71% in 2018 and 67% in 2019) and within populations (25% in 2018 and 28% in 2019) rather than among populations (3% in 2018 and 5% in 2019), but the among population-level variation was statistically significant (Table 3).

Prevalence and infection load of the apicomplexans

Apicomplexan infection prevalence and infection load patterns are summarized in Table 4. Regarding the *Rhytidocystis* sp. prevalence, the best-fitting model included only population-level differences; logistic regression showed that the probability of being infected differed between Cramond Beach and Vellerup (Wald $\chi^2 = 18.4$, $df = 1$, $p < 0.001$), while the Herslev population did not differ significantly from either (Table 5). The probability of being infected remained constant over the two sampling years (Wald $\chi^2 = 0.12$, $df = 1$, $p = 0.73$) (Figure 1A). Prevalence was very high overall: 93% of the studied hosts were infected. The highest prevalence was found at Cramond Beach, where 97% of hosts were infected in 2018 and 90% in 2019. In Herslev, 88% of hosts were infected in 2018 and 85% in 2019. Lowest prevalence was found in Vellerup in 2018, where 75% of the hosts were infected. Infection by *S. pygospionis* did not affect the probability of being infected by *Rhytidocystis* sp. (Wald $\chi^2 = 0.1$, $df = 1$, $p = 0.75$).

The prevalence of *S. pygospionis* infection also differed among the populations (Wald $\chi^2 = 50.7$, $df = 2$, $p < 0.001$), and the probability of being infected was lower in 2019 than in 2018 (Wald $\chi^2 = 5.0$, $df = 1$, $p = 0.026$) (Figure 1B, Table 5). The highest prevalence was again found at Cramond Beach, where 87% of the hosts were infected in 2018 and 53% in 2019, significantly higher than in the Danish populations (Table 5). The lowest prevalence was detected in Herslev, with 12% of hosts infected in 2018 and only one host (5%) in 2019. In Vellerup, the prevalence of *S. pygospionis* infection was 20% in both sampling years. The probability of being infected did not differ between the Danish populations (Wald's $\chi^2 = 2.3$, $df = 1$, $p = 0.13$) (Figure 1B). Additionally, an infection with *Rhytidocystis* sp. increased the probability of being infected with *S. pygospionis* (odds ratio: 1.2173) but the effect was not significant (Wald's $\chi^2 = 0.086$, $df = 1$, $p = 0.77$).

Infection by *Rhytidocystis* sp. was found to be highly aggregated, as variance-to-mean ratio was very high in all samples (Table 4). Linear regression analysis (Table 6) showed that *Rhytidocystis* sp. infection load differed between populations ($F = 19.00$, $df = 2$, $p < 0.001$). The highest mean infection load in both sampling times was found at Cramond Beach, being 651.17 copies/ng total DNA in 2018 and 380.62 copies/ng total DNA in 2019. In Herslev, the mean infection load was 367.2 copies/ng total DNA in 2018 and 152.5 copies/ng total DNA in 2019; and in Vellerup, 96.1 copies/ng total DNA in 2018 and 150.7 copies/ng total DNA in 2019 (Table 4, Figure 2A). There was no significant difference in *Rhytidocystis* sp. infection load between the sampling years ($F = 0.3003$, $df = 1$, $p = 0.585$).

We found that the infection load of *S. pygospionis* differed between the populations ($F = 34.717$, $df = 2$, $p < 0.001$), but not between the sampling years ($F = 1.05$, $df = 1$, $p = 0.3106$). The highest mean infection load was found at Cramond Beach, where it was 170.06 copies/ng total DNA in 2018 and 312.8 copies/ng total DNA in 2019. Infection was also highly aggregated within a few individuals (Table 4). In the Danish populations, *S. pygospionis* infection load was significantly lower than in Cramond Beach (Table 6), mean infection load being 2.52 copies/ng total DNA in Herslev and 1.00 copies/ng total DNA in Vellerup. Infection was also more evenly distributed within the sample, as the variance-to-mean ratio was less than the mean infection load (Table 4).

Out of a total of 179 hosts, 57 individuals (31.8%) were infected by both apicomplexans. Dual infections were more common at Cramond Beach than in the Danish populations ($\chi^2 = 60.822$, $df = 4$, $p < 0.001$) (Figure 3A). Infection loads of the two apicomplexans did not correlate with each other (Pearson's $r = 0.113$, $df = 53$, $p = 0.407$) (Figure 3B). However, when both apicomplexans coinfected the same host, *Rhytidocystis* sp. infection

TABLE 1 Genetic diversity for each sample

Sample	N	H_E	H_O	AR	F_{IS}
2018					
Cramond	27	0.424	0.344	5.511	0.228[a]
Herslev	31	0.412	0.321	4.192	0.172[a]
Vellerup	36	0.476	0.360	5.985	0.231[a]
2019					
Cramond	18	0.435	0.385	4.798	0.226[a]
Herslev	19	0.389	0.318	3.178	0.051
Vellerup	18	0.434	0.254	4.155	0.365[a]

Note: Expected heterozygosity and observed heterozygosity (H_E and H_O) were calculated using GenAlEx v. 6.501. Allelic richness (AR) and inbreeding coefficient (F_{IS}) were calculated using FSTAT v. 2.9.4. N = number of host individuals.

[a]F_{IS} deviating from zero significantly ($p < 0.05$).

load was significantly higher than single-species infection (F = 11.19, df = 1, p = 0.001) (Figure 3C). As most of the hosts that were infected by *S. pygospionis* were also infected by *Rhytidocystis* sp., no difference was found in *S. pygospionis* infection load when comparing dual infection to single-species infection (F = 0.141, df = 1, p = 0.708) (Figure 3D).

Host genetic diversity and infection patterns

Correlations between population genetic variability (F_{IS} and AR) and infection loads of both apicomplexans were not significant (Table 7). Individual heterozygosity did not differ between the populations (ANOVA: $F_{[1,92]}$ = 0.865, p = 0.355) or sampling years (ANOVA: $F_{[1,92]}$ = 0.089, p = 0.766). We chose to analyze the relationship between individual genetic diversity and the infection pattern of both apicomplexans using data only from Cramond Beach and Herslev populations due to significant deficiency of heterozygotes in Vellerup in 2019 (see Table 1). Logistic regression showed that hosts with higher heterozygosity had a higher probability of being infected by *Rhytidocystis* sp., but this association was not significant (odds ratio = 4.609, p = 0.439) (Figure 4A). Similarly, a nonsignificant association was found between individual heterozygosity and the probability of being infected by *S. pygospionis* (odds ratio = 3.193, p = 0.324) (Figure 4B).

Rhytidocystis sp. infection load was weakly negatively correlated with host heterozygosity, but the correlation was not significant (Pearson's r = −0.175, df = 83, p = 0.109) (Figure 5A). In contrast, correlation between *S. pygospionis* infection load and host heterozygosity was weakly positive, but again not significant (Pearson's r = 0.204, df = 34, p = 0.232) (Figure 5B). Heterozygosity did not differ between hosts that were infected by both apicomplexans compared with single infections ($F_{[1,133]}$ = 3.865, p = 0.0514) (Figure 6).

TABLE 2 Genetic differentiation between the populations calculated by pairwise F_{ST} values (above diagonal) and their significance (below diagonal)

	Cramond	Herslev	Vellerup
2018			
Cramond	–	0.0743	0.0078
Herslev	*	–	0.0325
Vellerup	NS	*	–
2019			
Cramond	–	0.1174	0.0477
Herslev	*	–	0.0086
Vellerup	NS	NS	–

Abbreviation: NS, nonsignificant.
*p<0.05.

DISCUSSION

In this study, we examined coinfection patterns of two marine apicomplexan species, *Rhytidocystis* sp. and *Selenidium pygospionis,* in natural host populations, and whether these interactions are associated with host population genetic variation and individual genetic diversity. Assuming all species within the phylum Apicomplexa are parasitic, we would expect that population genetic diversity and higher individual host heterozygosity would be associated with lower prevalence and infection loads; that is more heterozygous hosts would be less susceptible to infection and more diverse populations would have lower infection loads. In contrast to our expectation, we did not find any such association. Genetic diversity measured by allelic richness and inbreeding coefficient was not correlated with either species infection load and did not differ between the populations. The prevalence

TABLE 3 Analysis of molecular variance (AMOVA) for 94 hosts in 2018 and 55 hosts in 2019 in the three populations based on 9999 permutations

	df	SS	Variance component	Total variance [%]	F_{ST}
2018					
Among populations	2	10.318	0.052	3	0.033***
Among individuals	91	175.134	0.398	25	
Within individuals	94	106.000	1.128	71	
Total	187	291.452	1.578	100	
2019					
Among populations	2	9.006	0.071	5	0.046***
Among individuals	52	99.613	0.435	28	
Within individuals	55	57.500	1.045	67	
Total	109	166.118	1.551	100	

Note: Statistical significance of F_{ST} is based on standard permutation across the full dataset.
Abbreviations: *df*, degrees of freedom; SS, sum of squares.
***p<0.001.

TABLE 4 Summary of infection patterns in each studied *Pygospio elegans* populations

Sample	*N*	Infected (*Rhytidocystis* sp.)	Mean infection load (SE)[a]	$\sigma^2/\bar{x}$[a]	Infected (*S. pygospionis*)	Mean infection load (SE)[a]	$\sigma^2/\bar{x}$[a]
2018							
Cramond	39	38	651.2 (219.2)	2799.2	34	170.06 (44.13)	389.4
Herslev	40	35	367.2 (247.9)	5843.0	5	0.43 (0.1)	0.1
Vellerup	40	30	96.1 (46.5)	668.7	8	0.28 (0.1)	0.2
2019							
Cramond	20	18	380.6 (121.1)	692.0	10	312.8 (248.4)	1972.5
Herslev	20	17	152.5 (69.0)	527.1	1	13.0 (–)[b]	-
Vellerup	20	18	150.7 (96.5)	1104.0	4	0.31 (0.01)	0.0

Note: Total sample size (*N*), number of hosts infected by *Rhytidocystis* sp., mean infection load (mean number of *Rhytidocystis* sp. *cox1* copies per ng of total DNA), variance-to-mean ratio ($\sigma^2/\bar{x}$), number of hosts infected by *Selenidium pygospionis*, mean infection load (mean number of *S. pygospionis cox1* copies per ng of total DNA), and variance-to-mean ratio.

[a]Calculated with only infected hosts, SE = standard error of the mean.

[b]Only one host infected by *S. pygospionis*, SE, or variance-to-mean ratio cannot be calculated.

TABLE 5 Logistic regression for the best-fitting model odds ratios for prevalence of infection by the two apicomplexans

	OR (95% CI)	*Z*	*p*
Final GLM for prevalence of *Rhytidocystis* sp.			
Intercept	18.667 (6.901–76.567)	4.939	<0.001
Population			
Herslev	0.348 (0.074–1.276)	−1.499	0.134
Vellerup	0.214 (0.047–0.722)	−2.283	0.023
Final GLM for the prevalence of *S. pygospionis*			
Intercept	4.651 (2.338–10.239)	4.121	<0.001
Population			
Herslev	0.031 (0.010–0.084)	−6.340	<0.001
Vellerup	0.071 (0.027–0.170)	−5.684	<0.001
Year			
2019	0.361 (0.141–0.858)	−2.229	0.0258

Note: The distribution error function is binomial with a logistic link function. The references for population and year were Cramond Beach and 2018, respectively. Altogether 179 samples were utilized in this model, including 17–38 hosts per population and year.

Abbreviation: OR, odds ratio.

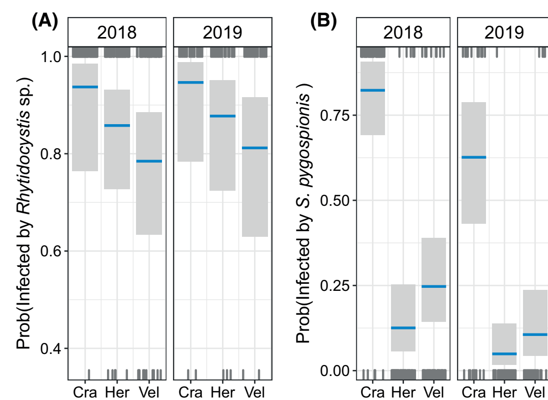

FIGURE 1 Prevalence of infection in the studied *Pygospio elegans* populations. (A) Probability of infection by *Rhytidocystis* sp. and (B) *Selenidium pygospionis*. Blue line indicates the expected value of prevalence from the logistic regression and gray band is a 95% confidence interval for the expected value. Upper and lower ticks represent the number of positive and negative residuals of the logistic regression, respectively

of infection was not affected by host individual heterozygosity, and neither apicomplexan infection loads were correlated with individual heterozygosity. Furthermore, host individual heterozygosity did not differ between hosts that were infected by only one species compared with hosts that were infected by both apicomplexans. To our knowledge, no previous research has focused on marine apicomplexan infection or coinfection in relation to their host's genetic diversity. However, in other invertebrate host–apicomplexan systems, varied relationships were found: Hosts with low individual genetic diversity had higher apicomplexan infection load in a damselfly (Kaunisto et al., 2013), but no association was found between host genetic diversity and infection in an earthworm (Velavan et al., 2009). Recently, it has also been suggested that correlations between host genetic diversity and parasite infection can be weak and nonsignificant when parasite virulence and fecundity are low (Lively et al., 2021), which could be the case in our study system.

While host population genetic diversity and individual heterozygosity did not differ between the sample sites, we detected significant population specific variation in infection patterns of the two apicomplexans. *Rhytidocystis* sp. prevalence was high in all studied populations (93%), but it was significantly lower in Vellerup than in Cramond Beach. *Selenidium pygospionis* prevalence was lower overall (34.8%), but significantly higher at Cramond Beach than in both Danish populations. *Rhytidocystis* sp. prevalence also remained constant

TABLE 6 Linear regression coefficients for the apicomplexan infection load

	Estimate (SE)	*t*	*p*
Linear model for *Rhytidocystis* sp. infection load ($r^2 = 0.240$, $F_{[2,153]} = 24.13$, $p < 0.001$)			
Intercept	5.049 (0.323)	15.613	<0.001
Population			
Herslev	−2.386 (0.466)	−5.121	<0.001
Vellerup	−3.120 (0.476)	−6.555	<0.001
Linear model for *S. pygospionis* infection load ($r^2 = 0.562$, $F_{[4,57]} = 18.29$, $p < 0.001$)			
Intercept	3.5590 (0.361)	11.679	<0.001
Population			
Herslev	−4.0279 (0.880)	−4.579	<0.001
Vellerup	−5.0069 (0.658)	−7.606	<0.001
Year			
2019	0.6137 (0.600)	1.023	0.311
Rhytidocystis sp. infection load	0.0003 (0.0002)	1.364	0.178

Note: The reference for population was Cramond Beach. The dependent variable in both models has been log-transformed, and distribution error function is normal. Because only infected hosts were used in the analysis, the sample size was 156 for *Rhytidocystis* sp. and 62 for *S. pygospionis*.

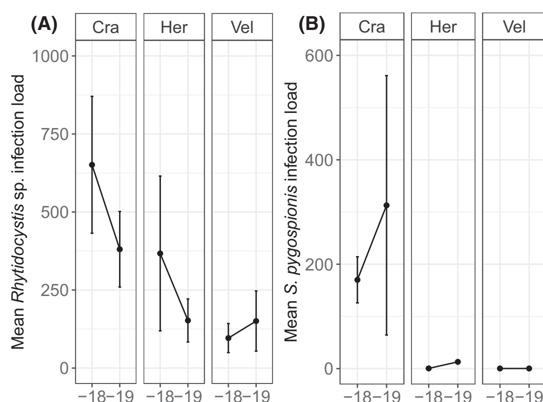

FIGURE 2 Mean infection load (*cox1* copies/ng total DNA) of the apicomplexans in the three host populations over the sampling period. (A) Mean infection load of *Rhytidocystis* sp. and (B) *S. pygospionis*. Error bars indicate the standard error of the mean

over the sampling years, while the proportion of hosts infected by *S. pygospionis* was lower in 2019 than in 2018. Similarly, infection loads of both species were significantly higher at Cramond Beach than those of the Danish populations. Differences in prevalence, infection loads, and parasite distribution patterns at population level suggest variation in local exposure to infective stages (Hansen et al., 2004; Karvonen et al., 2004). Exposure rates are affected by a variety of abiotic and biotic factors that affect the transmission of the infective stages, such as the local environmental factors (Poulin, 2013). The studied sites differ in multiple environmental conditions. For example, the Cramond Beach population located in the Firth of Forth, Scotland, faces two cycles of low and high tide within a day, whereas the Danish populations, located in Isefjord–Roskilde fjord, are not subjected to tidal currents. Tidal currents can strongly influence the diversity of the benthic communities (Warwick & Uncles, 1980), and high velocity of currents can increase parasite prevalence locally, enhancing transmission (Alaliyat et al., 2019; Correia et al., 2021; Halliday-Isaac et al., 2021). Marine apicomplexans transmit passively via an oral–fecal route (Leander, 2008), and the oocysts containing the infective stages can be highly persistent in the environment (Clopton et al., 2016). In our study, the apicomplexan exposure and transmission between hosts could be enhanced at Cramond Beach by tidal currents accumulating oocysts within tidal pools, leading to the higher observed prevalence and infection loads.

Another environmental factor that differs between the studied sites and could potentially affect the exposure rate is salinity. Salinity measured at Cramond Beach was 31 ppt in 2018 and 32 ppt in 2019, while in the Danish populations, it ranged from 14 to 18 ppt, being lowest in Herslev. Marine species diversity has been shown to increase within salinity values that are considered as optimal (Clavero et al., 2000; Montagna et al., 2002); therefore, the observed differences in prevalence between sites could also be due to differing optimal salinity ranges for the two apicomplexans. Also, our study is the first to report *S. pygospionis* in the Baltic Sea, and low prevalence of *S. pygospionis* in the Danish populations suggests that given the differences in the environmental conditions and geographical distance between the studied sites, it is possible that *S. pygospionis* has not yet fully managed to colonize the Danish *P. elegans* populations.

The same factors that could affect differences in apicomplexan prevalence and infection loads could also cause differences in the abundance of coinfections (Karvonen et al., 2019). Overall, we detected concurrent infections in 32% of hosts. Concurrent infections were significantly more common in the Cramond Beach population, where no symbiont-free hosts were observed (all sampled hosts were either infected with one or both species). Almost all hosts that were infected by *S. pygospionis* were also infected by *Rhytidocystis* sp. When both apicomplexans coinfected the same host, the *Rhytidocystis* sp. infection load was significantly higher than single-species infection load, but no significant change was found in *S. pygospionis,* suggesting possible facilitation provided by *S. pygospionis*. Facilitation could occur, for example, by suppression of the host immune system, as has been suspected of parasitic mites and wing deforming virus in honeybees (Nazzi et al., 2012) and myxoma virus and the nematode *Trichostrongylus retortaeformis* infections in rabbits (Cattadori et al., 2007). Coinfecting parasites could, alternatively, antagonize each other within the host by competing for space or energetic resources (Clerc et al., 2019; Ezenwa & Jolles, 2011;

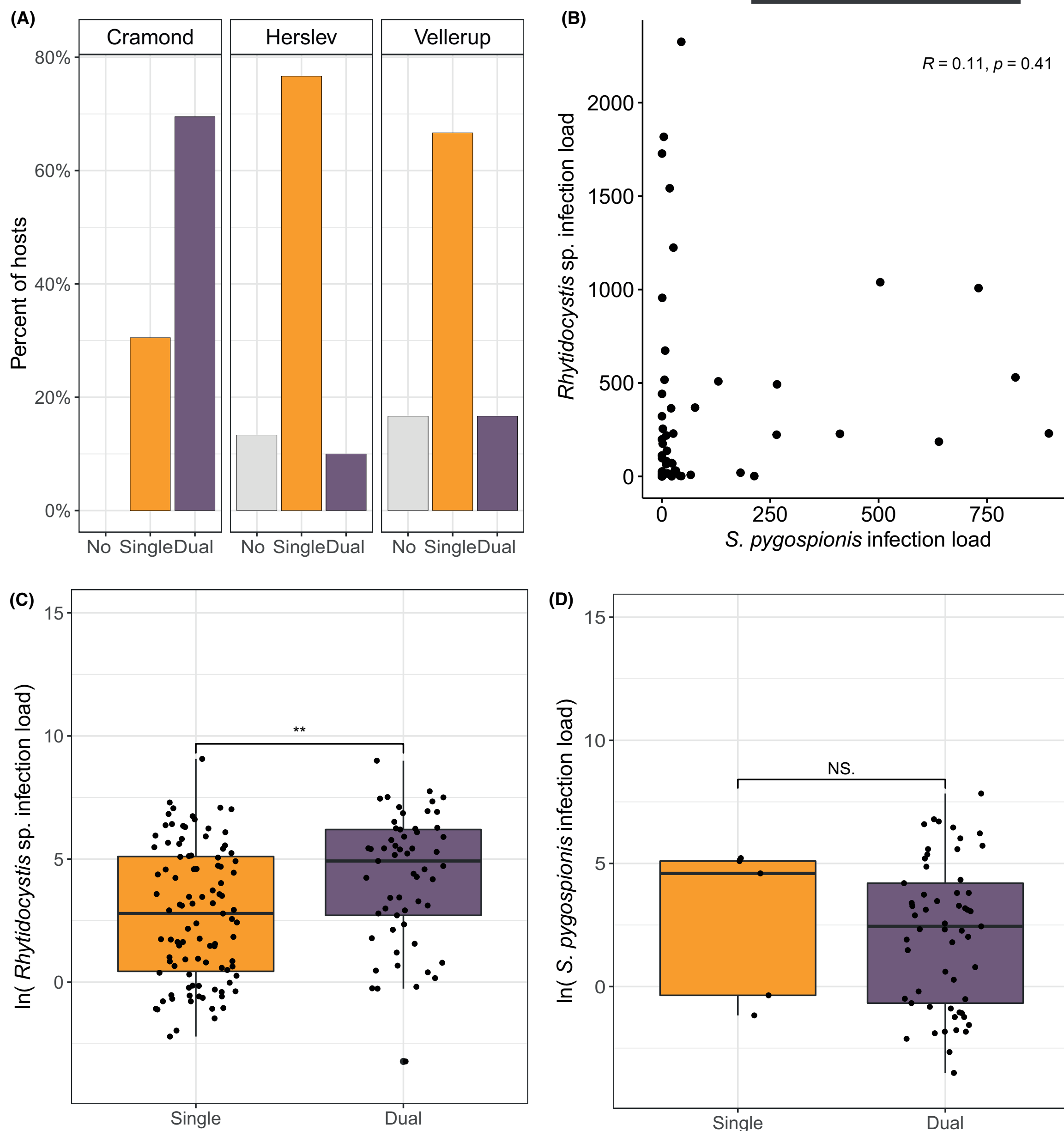

FIGURE 3 Coinfection patterns of *Rhytidocystis* sp. and *Selenidium pygospionis*. (A) Proportion of dual- and single-infected hosts and hosts with no infection in the whole dataset ($N = 176$). (B) Nonsignificant positive correlation between infection loads of the apicomplexans within hosts infected by both apicomplexans ($N = 55$). (C) Dual infections had higher *Rhytidocystis* sp. infection load than single infections. The solid line depicts the median and $^{**}p < 0.001$. (D) No difference was found in the infection load of *S. pygospionis* between hosts with single infection and dual infection. NS = nonsignificant difference

Graham, 2008). The apicomplexans studied here infect the intestine of *P. elegans* (Hiillos et al., 2021; Paskerova et al., 2018), a circumstance that could lead to a negative outcome through competition of host resources (Dallas et al., 2019). However, as dual infections were relatively frequent, and *Rhytidocystis* sp. infection load was higher in concurrent infections, synergistic interactions between the apicomplexan species seem more likely. Another possibility is that *Rhytidocystis* sp. could gain some fitness advantage from *S. pygospionis* when infecting the same host. This could be due to priority effects, that is described for parasites, when the order in which parasites that infect their hosts can lead to positive outcome for one of the parasites even when they antagonize each other within the host (Clay et al., 2019a, 2019b; Lohr et al., 2010). For example, a dominant trematode

Echinostoma caproni infecting a freshwater snail was found to have higher reproductive output when the host was first infected by another trematode, *Schistosoma mansoni* (Carpenter et al., 2021). Controlled experiments or observations on the apicomplexan fitness changes (e.g. oocyst production) within coinfected hosts would be required to confirm whether priority effects are occurring in this system. Additionally, coinfections tend to be temporally dynamic (Karvonen et al., 2019), meaning that two symbionts are more likely to infect the host sequentially rather than simultaneously and they can exhibit different seasonal cycles of infection (Grunberg & Sukhdeo, 2017). In our previous study, we found that *Rhytidocystis* sp. infection is seasonally dynamic, with infection load being high in fall and declining in spring (Hiillos et al., 2021). Whether *S. pygospionis* has a seasonal pattern of infection is unknown, but a different seasonal cycle could explain the observed differences in prevalence and infection loads for the two species.

TABLE 7 Pearson's correlation between measures of inbreeding coefficient, allelic richness, and estimates of infection loads of *Rhytidocystis* sp. and *S. pygospionis*

Measures	*r*	*df*	*p*
Rhytidocystis sp.			
Inbreeding coefficient	0.0102	4	0.9847
Allelic richness	0.2836	4	0.5860
S. pygospionis			
Inbreeding coefficient	0.0744	4	0.8885
Allelic richness	0.2709	4	0.6036

Interestingly, *Polyrhabdina pygospionis*, a third symbiont known to infect *P. elegans,* has been documented in frequent coinfections with *S. pygospionis,* with higher infection loads in concurrent infections than in single-species infections (Paskerova et al., 2018, 2021). Those observations are similar to what we observed between *Rhytidocystis* sp. and *S. pygospionis* in this study, suggesting that *S. pygospionis* could facilitate a variety of symbiotic relationships in the host. Quantification of *P. pygospionis* in addition to the species studied here could help resolve the interactions of these coinfecting apicomplexans within their host, and empirical laboratory experiments would be required to confidently confirm whether facilitation is occurring between these species.

Although our expectations for the relationship between host genetic diversity and infection patterns were based on theory describing host–parasite interactions, it is possible that the studied apicomplexans are not actually parasites, but rather harmless commensals, or that have a beneficial relationship with their hosts, as has been suggested for the gregarines (Rueckert et al., 2019). For example, in invertebrates, it has been demonstrated that gregarine infections can be beneficial for their hosts (Alarcón et al., 2017; Arcila & Meunier, 2020; Bollatti & Ceballos, 2014; Valigurová, 2012) and some have even been suggested to be essential for their hosts (Sumner, 1936). The infection patterns observed in our study reflect those of typical parasite infections for *S. pygospionis*; varying prevalence between populations is a common characteristic to parasitic apicomplexans, and for *Rhytidocystis* sp., the infection patterns observed in our

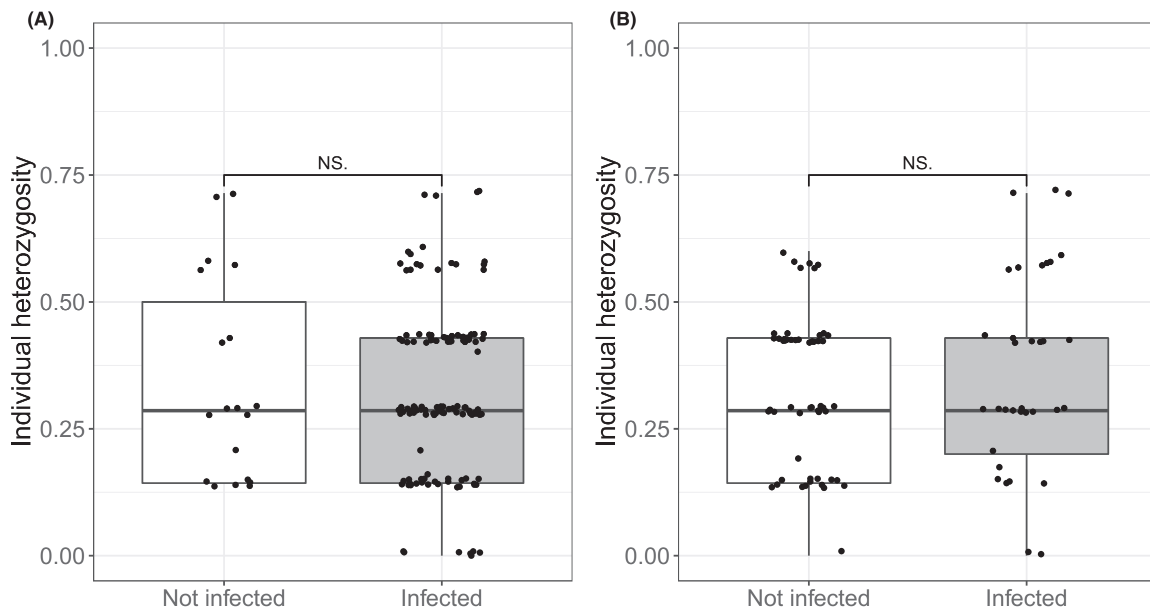

FIGURE 4 No association was detected between host individual heterozygosity and prevalence of infection by (A) *Rhytidocystis* sp. and (B) *Selenidium pygospionis*. The solid line depicts the median. NS = nonsignificant

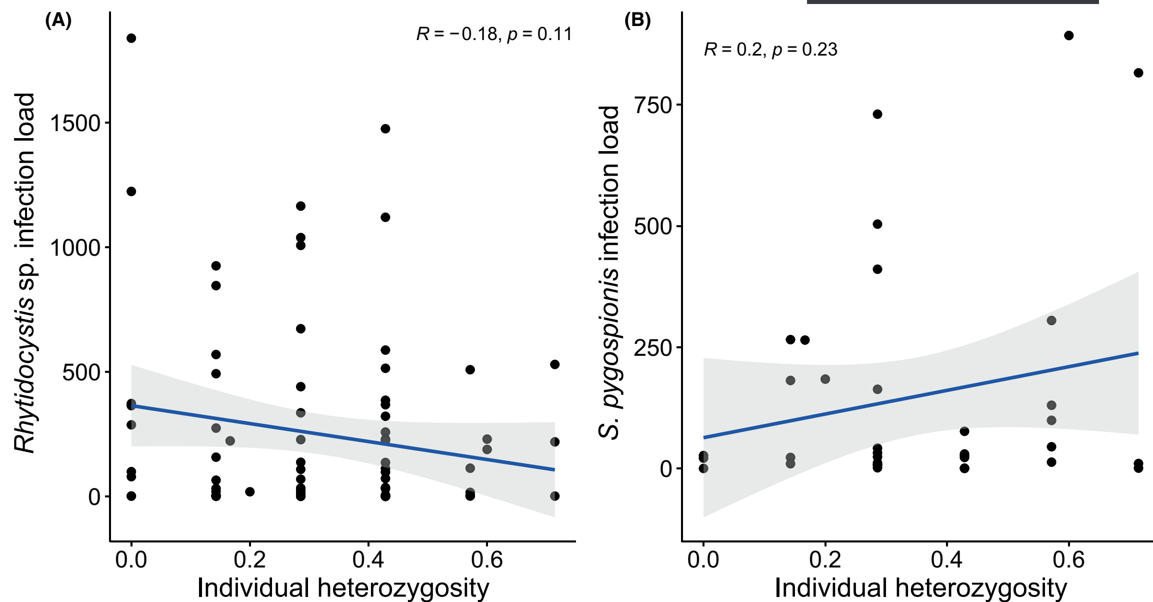

FIGURE 5 (A) Nonsignificant negative correlation between individual heterozygosity and *Rhytidocystis* sp. infection load ($N = 85$) and (B) nonsignificant positive correlation between individual heterozygosity and *S. pygospionis* infection load ($N = 36$). Blue regression line notes the direction of correlation, and gray area shows the 95% confidence interval level

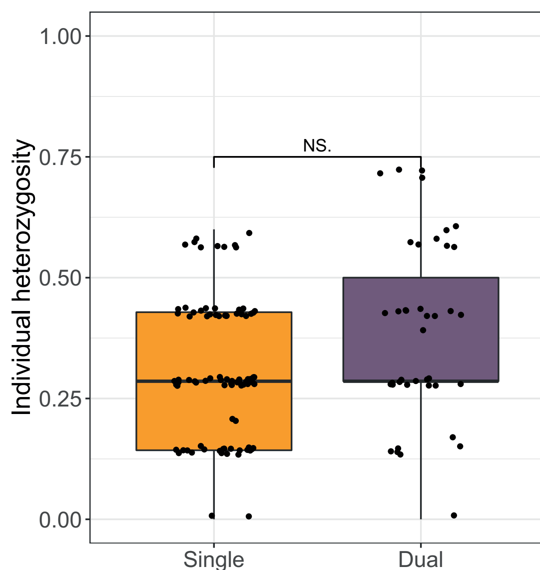

FIGURE 6 Individual heterozygosity for hosts with single-species infection ($N = 92$) and dual infection ($N = 43$). NS = nonsignificant difference between single infection vs. dual infection

study reflect those of beneficial symbionts, which are often found in all hosts (Saffo, 1982; Saffo et al., 2010). Their aggregated distributions, where few hosts harbor most symbionts, are typical for parasitic interactions (Anderson & May, 1978; Poulin, 1993). Nevertheless, additional research will be needed to determine how the studied symbionts affect host fitness.

If the apicomplexans studied here are not harmful to their host, our expectations that higher genetic variation at population and individual levels is associated with lower apicomplexan prevalence and infection load might be incorrect. Even if the expectation is correct, some technical issues could have prohibited us finding an association between host genetic diversity and apicomplexan infection. Firstly, we used neutrally evolving microsatellite markers to assess individual host heterozygosity (Kesäniemi et al., 2012; Thonig et al., 2017); however, we did not measure heterozygosity–fitness correlations. Hence, it is possible that diversity in these loci does not correlate with fitness, and they might not reflect susceptibility to infection. In addition, correlation between microsatellite heterozygosity and genomic heterozygosity is suggested to be low when less than ten markers are used (DeWoody & DeWoody, 2005). Although heterozygosity at microsatellite loci has been positively correlated with resistance to parasites in many studies (Acevedo-Whitehouse et al., 2003; Coltman et al., 1999; Isomursu et al., 2012), contrasting results have also been documented (Velavan et al., 2009). Microsatellites located in candidate genes associated with resistance to infections would be more appropriate for linking heterozygosity and parasite infection (Luikart et al., 2008) if such markers could be identified for *P. elegans*. Secondly, the effects of host microsatellite heterozygosity on parasite infection might depend on the infecting species (Isomursu et al., 2012; Portanier et al., 2019). Portanier et al. (2019)

found that resistance to gastrointestinal nematodes is associated with both neutral and adaptive genetic diversity in Mediterranean mouflon, but no association was found between coccidian parasite burden and genetic diversity (Portanier et al., 2019). Similarly, Isomursu et al. (2012) found that microsatellite heterozygosity was correlated with nematode infection, but not with cestode infection in capercaillie.

Furthermore, the small sample sizes might not reflect the populations as well as we intended. We found positive F_{IS} values in all populations, indicating that samples are not in the Hardy–Weinberg equilibrium and could have experienced significant inbreeding. The deficiency of heterozygotes observed could be a consequence of limited sampling. As *P. elegans* is known to have patchy distributions (Bolam, 2004), a high number of related individuals could also have been sampled due to chance. If the F_{IS} values indeed reflect inbreeding, it is important to keep in mind that several studies have shown that inbreeding depression can increase susceptibility to infection in vertebrates (Acevedo-Whitehouse et al., 2003; Coltman et al., 1999) and in invertebrates (Whitehorn et al., 2011), but the outcome is not always straightforward (Puurtinen et al., 2004; Stevens et al., 1997). Our analysis did not indicate any such association, as measures of F_{IS} were not correlated with infection of either apicomplexan species.

Finally, it is important to note that the use of *cox1* to estimate infection load does not indicate the absolute number of either of the studied symbionts. The copy number of *cox1* in these species is currently not known, and therefore, the infection load estimates should be taken cautiously. *Selenidium pygospionis*, such as many other archigregarines, is known to possess multiple mitochondria, and the number of mitochondria is thought to increase as the cell grows (Desportes & Schrével, 2013; Leander, 2006; Paskerova et al., 2018). Likewise, species in the genus *Rhytidocystis* are also known to harbor more than one mitochondrion (Leander & Ramey, 2006). Therefore, to be able to obtain more precise infection load estimates, a single-copy nuclear marker would be more appropriate. Nevertheless, since the *cox1* gene is currently the only available marker for *Rhytidocystis* sp., and it can reliably be used to distinguish the studied symbionts from each other and their host (Hiillos et al., 2021), the used method still provides estimates of the infection loads and allows the investigation of coinfection patterns of these two species.

CONCLUSION

Our results suggest that factors other than host genetic diversity might be more important in determining infection dynamics of the studied marine apicomplexans in their polychaete host. Both apicomplexans showed population-specific infection patterns. Concurrent infections were common but varied between the sites (Scotland vs. Denmark), suggesting that differences in encounter rates, rather than differences in susceptibility, might be a primary explanation. On the contrary, the effect of host genetics on these symbionts is potentially masked by a complex interaction network between the different apicomplexan species (including *P. pygospionis* not studied here). It is not currently known whether the symbionts are causing any harm to their host; hence, parasite-mediated selection pressure might not be occurring. Further studies are required to resolve the nature of the interaction between the studied apicomplexans and their host, as well as how they interact with each other during coinfection.

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III

BENTHIC COMMUNITY DIVERSITY INDICATES DIVERSITY OF MARINE APICOMPLEXA IN COASTAL SITES OF THE BALTIC SEA, BUT DILUTION EFFECTS MIGHT DRIVE INFECTION PATTERNS

by

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