Received: 12 April 2022 | Revised: 6 June 2022 | Accepted: 7 June 2022

DOI: 10.1111/jeu.12932

ORIGINAL ARTICLE

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

Anna-Lotta Hiillos[1] | **Irin Rony**[1] | **Sonja Rueckert**[2,3] | **K. Emily Knott**[1]

[1]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

[2]School of Applied Sciences, Edinburgh Napier University, Edinburgh, UK

[3]Centre for Conservation and Restoration Science, Edinburgh Napier University, Edinburgh, UK

Correspondence
Anna-Lotta Hiillos, Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland.
Email: anna-lotta.l.m.hiillos@jyu.fi

Funding information
Gordon and Betty Moore Foundation; Ellen and Artturi Nyyssönen Foundation; Erasmus+ Travel Grant; Emil Aaltonen Foundation Grant, Grant/Award Number: a6a412; University of Jyväskylä Graduate School

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

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 $\chi^2 = 0.1$, $df = 1$, $p = 0.75$).

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 ($p < 0.05$).

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

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.

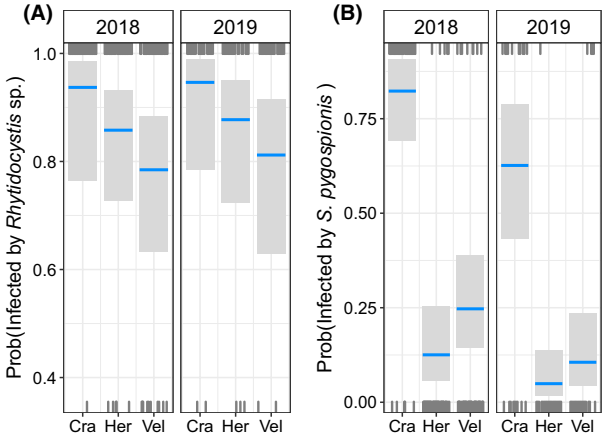

FIGURE 1 Prevalence of infection in the studied *Pygospio elegans* populations. (A) Probability of infection by *Rhytidocystis* sp. and (B) *Selenidium pygospionis*. Blue line indicates the expected value of prevalence from the logistic regression and gray band is a 95% confidence interval for the expected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

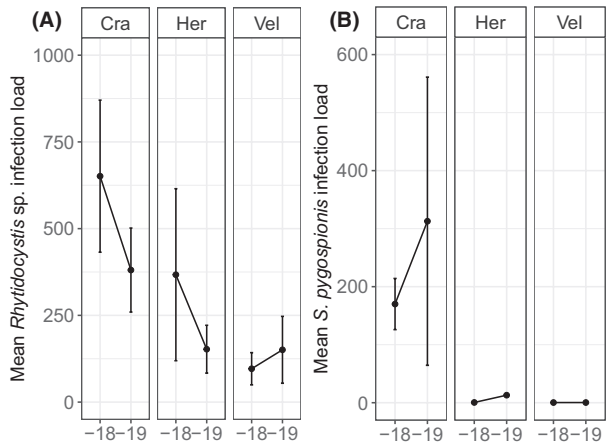

FIGURE 2 Mean infection load (*cox1* copies/ng total DNA) of the apicomplexans in the three host populations over the sampling period. (A) Mean infection load of *Rhytidocystis* sp. and (B) *S. pygospionis*. Error bars indicate the standard error of the mean

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;

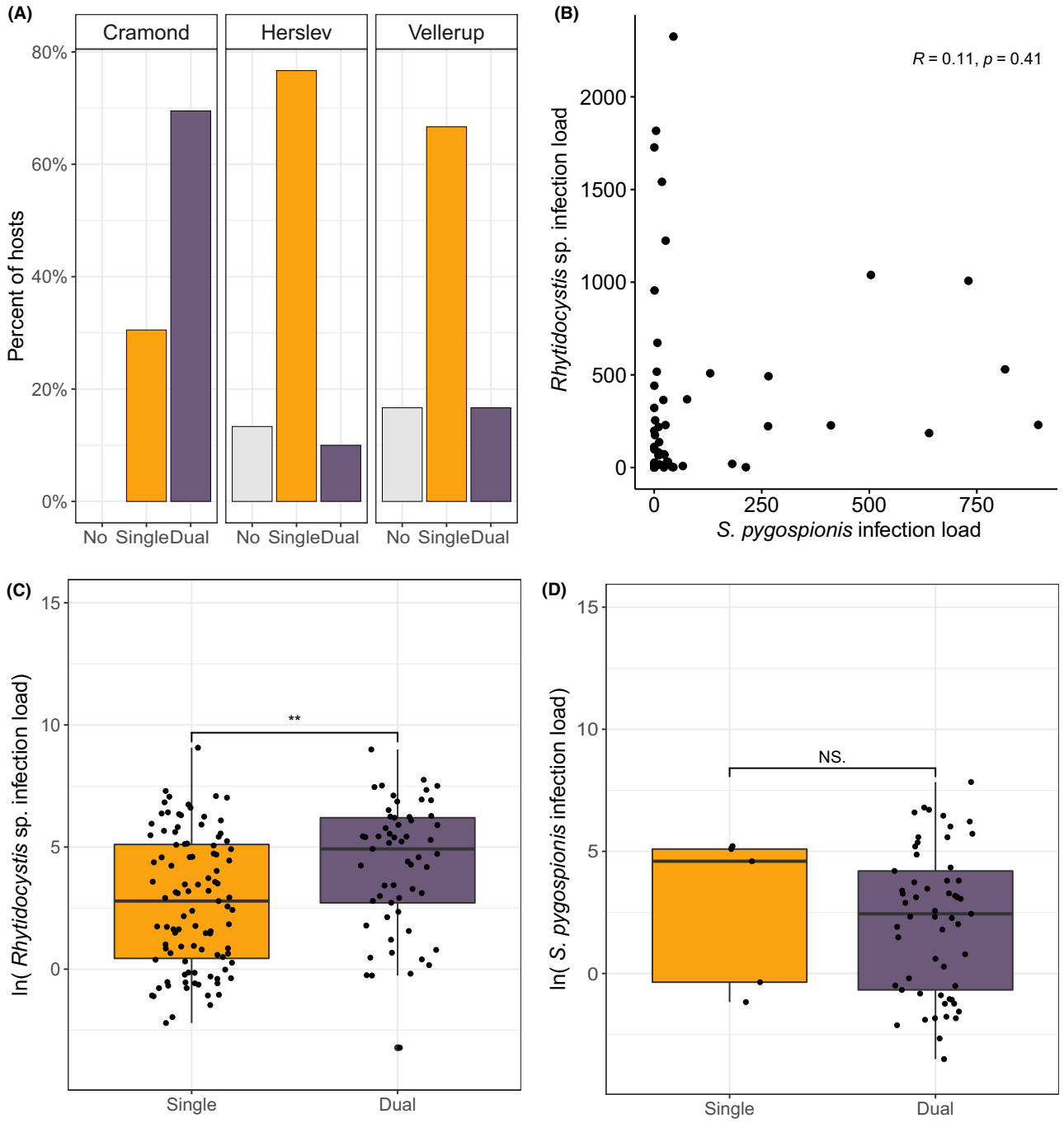

FIGURE 3 Coinfection patterns of *Rhytidocystis* sp. and *Selenidium pygospionis*. (A) Proportion of dual- and single-infected hosts and hosts with no infection in the whole dataset ($N = 176$). (B) Nonsignificant positive correlation between infection loads of the apicomplexans within hosts infected 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

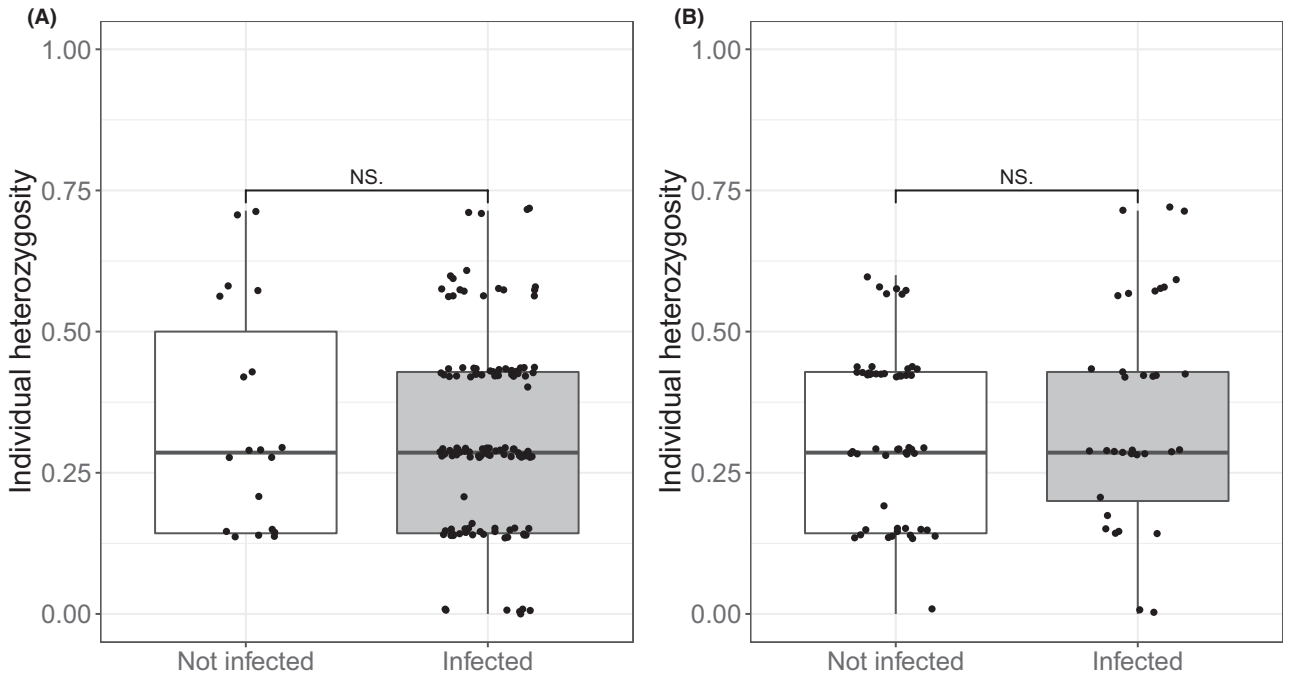

FIGURE 4 No association was detected between host individual heterozygosity and prevalence of infection by (A) *Rhytidocystis* sp. and (B) *Selenidium pygospionis*. The solid line depicts the median. NS = nonsignificant

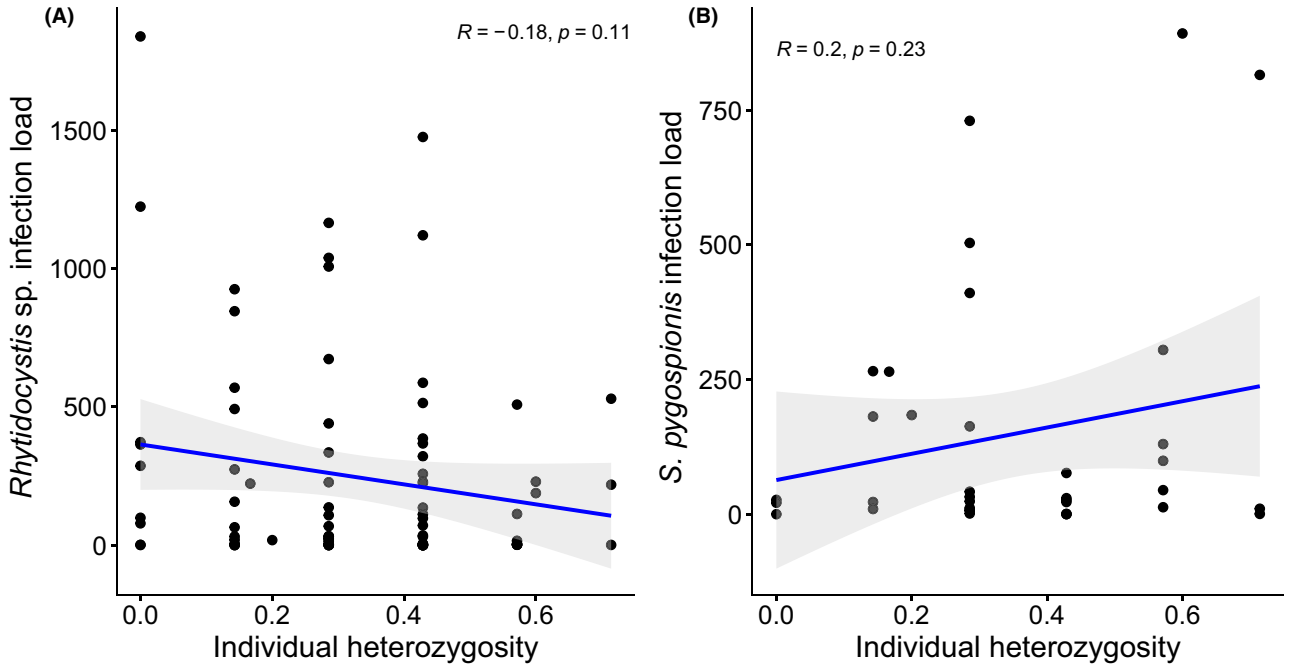

FIGURE 5 (A) Nonsignificant negative correlation between individual heterozygosity and *Rhytidocystis* sp. infection load ($N = 85$) and (B) nonsignificant positive correlation between individual heterozygosity and *S. pygospionis* infection load ($N = 36$). Blue regression line notes the direction of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

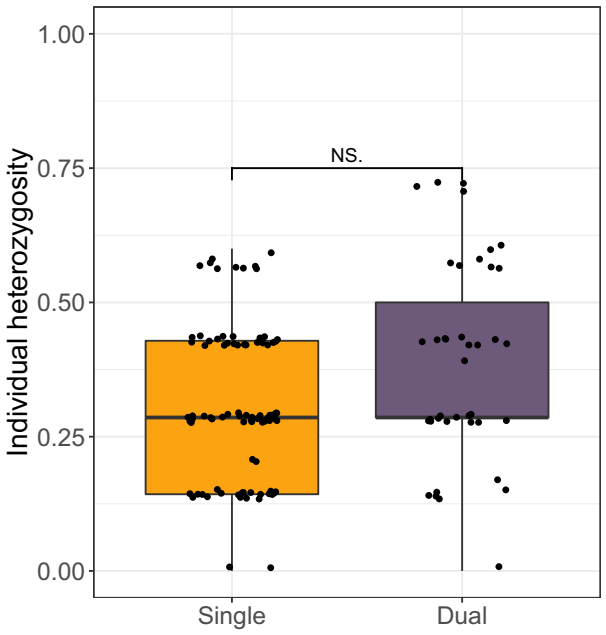

FIGURE 6 Individual heterozygosity for hosts with single-species infection ($N = 92$) and dual infection ($N = 43$). NS = nonsignificant difference between single infection vs. dual infection

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.

ACKNOWLEDGMENTS

This work was funded by the University of Jyväskylä Graduate School for Doctoral Studies (Department of Biological and Environmental Science), Emil Aaltonen Foundation Grant (a6a412), Erasmus+ Travel Grant, and Ellen and Artturi Nyyssönen Foundation Grant to ALH. SR was supported by a grant from the Gordon and Betty Moore Foundation (GBMF9327/https://doi.org/10.37807/GBMF9327). We would like to thank H. Cecilie Petersen, Benni W. Hansen, Gary Banta, and Anne Faborg at Roskilde University (DK) for the help in collecting samples from the Danish populations. We would also like to thank Minne Jartti for assisting in the laboratory and Gita Paskerova for providing gregarine samples used as positive controls in this study. Open access funding enabled and organized by ProjektDEAL.

ORCID

Anna-Lotta Hiillos https://orcid.org/0000-0002-8536-3331

REFERENCES

Acevedo-Whitehouse, K., Gulland, F., Greig, D. & Amos, W. (2003) Disease susceptibility in California Sea lions. *Nature*, 422, 35. https://doi.org/10.1038/422035a

Alaliyat, S., Yndestad, H. & Davidsen, P.I. (2019) An agent-based approach for predicting patterns of pathogen transmission between aquaculture sites in the Norwegian fjords. *Aquaculture*, 505, 98–111. https://doi.org/10.1016/j.aquaculture.2019.02.044

Alarcón, M.E., Jara-F, A., Briones, R.C., Dubey, A.K. & Slamovits, C.H. (2017) Gregarine infection accelerates larval development of the cat flea *Ctenocephalides felis* (Bouché). *Parasitology*, 144, 419–425. https://doi.org/10.1017/S0031182016002122

Alizon, S., de Roode, J.C. & Michalakis, J. (2013) Multiple infections and the evolution of virulence. *Ecology Letters*, 16, 556–567. https://doi.org/10.1111/ele.12076

Altermatt, F. & Ebert, D. (2008) Genetic diversity of *Daphnia magna* populations enhances resistance to parasites. *Ecology Letters*, 11, 918–928. https://doi.org/10.1111/j.1461-0248.2008.01203.x

Amos, W., Worthington Wilmer, J., Fullard, K., Burgh, T.M., Croxall, J.P., Bloch, D. et al. (2001) The influence of parental relatedness on reproductive success. *Proceedings of the Royal Society of London B*, 268, 2021–2027. https://doi.org/10.1098/rspb.2001.1751

Anderson, R.M. & May, R.M. (1978) Regulation and stability of host-parasite population interactions: I. Regulatory processes. *Journal of Animal Ecology*, 47, 219–247.

Anderson, R.M. & May, R.M. (1982) Coevolution of hosts and parasites. *Parasitology*, 85, 411–426.

Arcila, F. & Meunier, J. (2020) Friend or foe? The apparent benefits of gregarine (Apicomplexa: Sporozoa) infection in the European earwig. *International Journal for Parasitology*, 50, 461–469. https://doi.org/10.1016/j.ijpara.2020.01.007

Behnke, J.M., Eira, C., Rogan, M., Gilbert, F.S., Torres, J., Miquel, J. et al. (2009) Helminth species richness in wild wood mice, *Apodemus sylvaticus*, is enhanced by the presence of the intestinal nematode *Heligmosomoides polygyrus*. *Parasitology*, 136, 793–804. https://doi.org/10.1017/S0031182009006039

Bérénos, C., Wegner, K.M. & Schmid-Hempel, P. (2011) Antagonistic coevolution with parasites maintains host genetic diversity: an experimental test. *Proceedings of the Royal Society B*, 278, 218–224. https://doi.org/10.1098/rspb.2010.1211

Bolam, S.G. & Fernandes, T.F. (2003) Dense aggregations of *Pygospio elegans* (Claparède): effect on macrofaunal community structure and sediments. *Journal of Sea Research*, 49, 171–185. https://doi.org/10.1016/S1385-1101(03)00007-8

Bolam, S.G. (2004) Population structure and reproductive biology of *Pygospio elegans* (polychaeta: Spionidae) on an intertidal sandflat, Firth of Forth, Scotland. *Invertebrate Biology*, 123, 260–268.

Bollatti, F. & Ceballos, A. (2014) Effect of gregarines (Apicomplexa: Sporozoa) on survival and weight loss of *Victorwithius similis* Beier, 1959 (Arachnida: Pseudoscorpiones). *Journal of Invertebrate Pathology*, 117, 13–18. https://doi.org/10.1016/j.jip.2014.01.002

Cabalzar, A.P., Fields, P.D., Kato, Y., Watanabe, H. & Ebert, D. (2019) Parasite-mediated selection in a natural metapopulation of *Daphnia magna*. *Molecular Ecology*, 8, 4770–4785. https://doi.org/10.1111/mec.15260

Carpenter, S.A., Vannatta, J.T. & Minchella, D.J. (2021) Host exposure history and priority effects impact the development and reproduction of a dominant parasite. *International Journal for Parasitology*, 51, 935–943. https://doi.org/10.1016/j.ijpara.2021.03.007

Cattadori, I.M., Albert, R. & Boag, B. (2007) Variation in host susceptibility and infectiousness generated by co-infection: the myxoma–*Trichostrongylus retortaeformis* case in wild rabbits. *Journal of the Royal Society Interface*, 4, 831–840. https://doi.org/10.1098/rsif.2007.1075

Charpentier, M., Setchell, J.M., Prugnolle, F., Knapp, L.A., Wickings, E.J., Peignot, P. et al. (2005) Genetic diversity and reproductive success in mandrills (*Mandrillus sphinx*). *Proceedings of the National Academy of Sciences of the United States of America*, 102, 16723–16728. https://doi.org/10.1073/pnas.0507205102

Clay, P.A., Cortez, M.H., Duffy, M.A. & Rudolf, V.H.W. (2019a) Priority effects within coinfected hosts can drive unexpected population-scale patterns of parasite prevalence. *Oikos*, 128, 571–583. https://doi.org/10.1111/oik.05937

Clay, P.A., Dhir, K., Rudolf, V.H.W. & Duffy, M.A. (2019b) Within-host priority effects systematically alter pathogen coexistence. *The American Naturalist*, 193, 187–199. https://doi.org/10.1086/701126

Clavero, E., Hernández-Mariné, M., Grimalt, J.O. & Garcia-Pichel, F. (2000) Salinity tolerance of diatoms from thalassic hypersaline environments. *Journal of Phycology*, 36, 1021–1034. https://doi.org/10.1046/j.1529-8817.2000.99177.x

Clerc, M., Fenton, A., Babayan, S.A. & Pedersen, A.B. (2019) Parasitic nematodes simultaneously suppress and benefit from coccidian coinfection in their natural mouse host. *Parasitology*, 146, 1096–1106. https://doi.org/10.1017/S0031182019000192

Clopton, R.E., Steele, S.M. & Clopton, D.T. (2016) Environmental persistence and infectivity of oocysts of two species of gregarines, *Blabericola migrator* and *Blabericola cubensis* (Apicomplexa: Eugregarinida: Blabericolidae), parasitizing blaberid cockroaches (Dictyoptera: Blaberidae). *The Journal of Parasitology*, 102, 169–173. https://doi.org/10.1645/15-934

Coltman, D.W., Pilkington, J.G., Smith, J.A. & Pemberton, J.M. (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, Island population. *Evolution*, 53, 1259–1267. https://doi.org/10.2307/2640828

Correia, S., Picado, A., de Montaudouin, X., Freitas, R., Rocha, R.J.M., Dias, J.M. et al. (2021) Parasite assemblages in a bivalve host associated with changes in hydrodynamics. *Estuaries and Coasts: Journal of the Estuarine Research Federation*, 44, 1036–1049. https://doi.org/10.1007/s12237-020-00848-4

Dallas, T.A., Laine, A.-L. & Ovaskainen, O. (2019) Detecting parasite associations within multi-species host and parasite communities. *Proceedings of the Royal Society B: Biological Sciences*, 286, 20191109. https://doi.org/10.1098/rspb.2019.1109

Desportes, I. & Schrével, J. (Eds.). (2013) *Treatise on zoology – anatomy, taxonomy, biology. The gregarines (2 vols)*. Leiden, The Netherlands: Brill. https://doi.org/10.1163/9789004256057

de Meeûs, T. & Renaud, F. (2002) Parasites within the new phylogeny of eukaryotes. *Trends in Parasitology*, 18, 247–251. https://doi.org/10.1016/s1471-4922(02)02269-9

DeWoody, Y.D. & DeWoody, J.A. (2005) On the estimation of genome-wide heterozygosity using molecular markers. *The Journal of Heredity*, 96, 85–88. https://doi.org/10.1093/jhered/esi017

Dib, L., Bitam, I., Tahri, M., Bensouilah, M. & de Meeûs, T. (2008) Competitive exclusion between piroplasmosis and anaplasmosis agents within cattle. *PLoS Pathogens*, 4, e7. https://doi.org/10.1371/journal.ppat.0040007

Dobson, A. (1985) The population dynamics of competition between parasites. *Parasitology*, 91, 317–347.

Ekroth, A.K.E., Rafaluk-Mohr, C. & King, K.C. (2019) Host genetic diversity limits parasite success beyond agricultural systems: a meta-analysis. *Proceedings of the Royal Society B: Biological Sciences*, 286, 20191811. https://doi.org/10.1098/rspb.2019.1811

Elton, C.S. (1958) *The ecology of invasions by animals and plants*. London: Chapman & Hall.

Ezenwa, V.O. & Jolles, A.E. (2011) From host immunity to pathogen invasion: the effects of helminth coinfection on the dynamics of microparasites. *Integrative and Comparative Biology*, 51, 540–551. https://doi.org/10.1093/icb/icr058

Garrett, K.A. & Mundt, C.C. (1999) Epidemiology in mixed host populations. *Phytopathology*, 89, 984–990.

Gibson, A.K., Raverty, S., Lambourn, D.M., Huggins, J., Magargal, S.L. & Grigg, M.E. (2011) Polyparasitism is associated with increased disease severity in toxoplasma gondii-infected marine sentinel species. *PLoS Neglected Tropical Diseases*, 5, e1142. https://doi.org/10.1371/journal.pntd.0001142

Goudet, J. 2003. FSTAT (ver. 2.9.4), a program to estimate and test population genetics parameters. https://www2.unil.ch/popgen/softwares/fstat.htm. Updated from Goudet J., (1995).

Goudet, J. (1995) FSTAT (version 1.2): A computer program to calculate F-statistics. *The Journal of Heredity*, 86, 485–486.

Graham, A.L. (2008) Ecological rules governing helminth–microparasite coinfection. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 566–570. https://doi.org/10.1073/pnas.0707221105

Grunberg, R.L. & Sukhdeo, M.V.K. (2017) Temporal community structure in two gregarines (*Rotundula gammari* and *Heliospora longissima*) co-infecting the amphipod *Gammarus fasciatus*. *The Journal of Parasitology*, 103, 6–13. https://doi.org/10.1645/16-47

Haldane, J.B.S. (1949) Disease and evolution. *Ricerca Scientifica Suppl. A.*, 19, 68–76.

Halliday-Isaac, A.K., Robinson, J.B., Cruz-Rivera, E., Campbell, A.G. & Sikkel, P.C. (2021) Environmental correlates of prevalence of an intraerythrocytic apicomplexan infecting Caribbean damselfish. *Parasitologia*, 1, 69–82. https://doi.org/10.3390/parasitologia1020009

Hansen, F., Jeltsch, F., Tackmann, K., Staubach, C. & Thulke, H.-H. (2004) Processes leading to a spatial aggregation of *Echinococcus multilocularis* in its natural intermediate host *Microtus arvalis*. *International Journal for Parasitology*, 34, 37–44.

Hiillos, A.-L., Thonig, A. & Knott, K.E. (2021) Droplet digital PCR as a tool for investigating dynamics of cryptic symbionts. *Ecology and Evolution*, 11, 17381–17396. https://doi.org/10.1002/ece3.8372

Hindson, B.J., Ness, K.D., Masquelier, D.A., Belgrader, P., Heredia, N.J., Makarewicz, A.J. et al. (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*, 83, 8604–8610. https://doi.org/10.1021/ac202028g

Hindson, C.M., Chevillet, J.R., Briggs, H.A., Gallichotte, E.N., Ruf, I.K., Hindson, B.J. et al. (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nature Methods*, 10, 1003–1005. https://doi.org/10.1038/nmeth.2633

Isomursu, M., Rätti, L. & Helle, P. (2012) Susceptibility to intestinal parasites and juvenile survival are correlated with multilocus microsatellite heterozygosity in the capercaillie (*Tetrao urogallus*). *Ornis Fennica*, 89, 109–119.

Jaenike, J., Stahlhut, J.K., Boelio, L.M. & Unckless, R.L. (2010) Association between *Wolbachia* and *Spiroplasma* within *drosophila neotestacea*: an emerging symbiotic mutualism? *Molecular Ecology*, 19, 414–425. https://doi.org/10.1111/j.1365-294X.2009.04448.x

Karvonen, A., Hudson, P.J., Seppälä, O. & Valtonen, E.T. (2004) Transmission dynamics of a trematode parasite: exposure, acquired resistance and parasite aggregation. *Parasitology Research*, 92, 183–188. https://doi.org/10.1007/s00436-003-1035-y

Karvonen, A., Jokela, J. & Laine, A.-L. (2019) Importance of sequence and timing in parasite coinfections. *Trends in Parasitology*, 35, 109–118. https://doi.org/10.1016/j.pt.2018.11.007

Kaunisto, K.M., Viitaniemi, H.M., Leder, E.H. & Suhonen, J. (2013) Association between host's genetic diversity and parasite burden in damselflies. *Journal of Evolutionary Biology*, 26, 1784–1789. https://doi.org/10.1111/jeb.12177

Keesing, F., Holt, R.D. & Ostfeld, R.S. (2006) Effects of species diversity on disease risk. *Ecology Letters*, 9, 485–498. https://doi.org/10.1111/j.1461-0248.2006.00885.x

Kesäniemi, J.E., Boström, C. & Knott, K.E. (2012) New genetic markers reveal population genetic structure at different spatial scales in the opportunistic polychaete *Pygospio elegans*. *Hydrobiologia*, 691, 213–223. https://doi.org/10.1007/s10750-012-1075-3

Kesäniemi, J.E., Mustonen, M., Boström, C., Hansen, B.W. & Knott, K.E. (2014) Temporal genetic structure in a poecilogonous polychaete: the interplay of developmental mode and environmental stochasticity. *BMC Evolutionary Biology*, 14, 12. https://doi.org/10.1186/1471-2148-14-12

King, K.C. & Lively, C.M. (2012) Does genetic diversity limit disease spread in natural host populations? *Heredity*, 109, 199–203. https://doi.org/10.1038/hdy.2012.33

Leander, B.S. (2006) Ultrastructure of the archigregarine *Selenidium vivax* (Apicomplexa) – a dynamic parasite of sipunculid worms (host: *Phascolosoma agassizii*). *Marine Biology Research*, 2, 178–190. https://doi.org/10.1080/17451000600724395

Leander, B.S. & Ramey, P.A. (2006) Cellular identity of a novel small subunit rDNA sequence clade of apicomplexans: description of the marine parasite *Rhytidocystis polygordiae* n. sp. (host: *Polygordius* sp., polychaeta). *Journal of Eukaryotic Microbiology*, 53, 280–291. https://doi.org/10.1111/j.1550-7408.2006.00109.x

Leander, B.S. (2008) Marine gregarines: evolutionary prelude to the apicomplexan radiation? *Trends in Parasitology*, 24, 60–67. https://doi.org/10.1016/j.pt.2007.11.005

Lively, C.M., Xu, J. & Ben-Ami, F. (2021) Causation without correlation: parasite-mediated frequency-dependent selection and infection prevalence. *Biology Letters*, 17, 20210321. https://doi.org/10.1098/rsbl.2021.0321

Lohr, J.N., Yin, M. & Wolinska, J. (2010) Prior residency does not always pay off – co-infections in daphnia. *Parasitology*, 137, 1493–1500. https://doi.org/10.1017/S0031182010000296

Luikart, G., Pilgrim, K., Visty, J., Ezenwa, V.O. & Schwartz, M.K. (2008) Candidate gene microsatellite variation is associated with parasitism in wild bighorn sheep. *Biology Letters*, 4, 228–231. https://doi.org/10.1098/rsbl.2007.0633

Manzi, F., Halle, S., Seemann, L., Ben-Ami, F. & Wolinska, J. (2021) Sequential infection of *Daphnia magna* by a gut microsporidium followed by a haemolymph yeast decreases transmission of both parasites. *Parasitology*, 148, 1566–1577. https://doi.org/10.1017/S0031182021001384

Mathur, V., Kwong, W. K., Husnik, F., Irwin, N. A. T., Kristmundsson, Á., Gestal, C., Freeman, M., & Keeling, P. J. (2020). Phylogenomics identifies a new major subgroup of apicomplexans, marosporida class nov., with extreme apicoplast genome reduction. *Genome Biology and Evolution*, 13(2). https://doi.org/10.1093/gbe/evaa244

Mathur, V., Wakeman, K. & Keeling, P.J. (2021) Parallel functional reduction in the mitochondria of apicomplexan parasites. *Current Biology*, 31, 2920–2928. https://doi.org/10.1016/j.cub.2021.04.028

Mattila, J. (1997) The importance of shelter, disturbance and prey interactions for predation rates of tube-building polychaetes (*Pygospio elegans* [Claparéde]) and free-living tubificid oligochaetes. *Journal of Experimental Marine Biology and Ecology*, 218, 215–228. https://doi.org/10.1016/S0022-0981(97)00075-0

Montagna, P.A., Kalke, R.D. & Ritter, C. (2002) Effect of restored freshwater inflow on macrofauna and meiofauna in upper Rincon bayou, Texas, USA. *Estuaries*, 25, 1436–1447. https://doi.org/10.1007/BF02692237

Morrison, D.A. (2009) Evolution of the Apicomplexa: where are we now? *Trends in Parasitology*, 25, 375–382. https://doi.org/10.1016/j.pt.2009.05.010

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G., Varricchio, P. et al. (2012) Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathogens*, 8, e1002735. https://doi.org/10.1371/journal.ppat.1002735

Ostfeld, R.S. & Keesing, F. (2012) Effects of host diversity on infectious disease. *Annual Review of Ecology, Evolution, and Systematics*, 43, 157–182. https://doi.org/10.1146/annurev-ecolsys-102710-145022

Paskerova, G.G., Miroliubova, T.S., Diakin, A., Kováčiková, M., Valigurová, A., Guillou, L. et al. (2018) Fine structure and molecular phylogenetic position of two marine gregarines, *Selenidium pygospionis* sp. n. and *S. pherusae* sp. n., with notes on the phylogeny of Archigregarinida (Apicomplexa). *Protist*, 169, 826–852. https://doi.org/10.1016/j.protis.2018.06.004

Paskerova, G.G., Miroliubova, T.S., Valigurová, A., Janouškovec, J., Kováčiková, M., Diakin, A. et al. (2021) Evidence from the resurrected family Polyrhabdinidae Kamm, 1922 (Apicomplexa: Gregarinomorpha) supports the epimerite, an attachment organelle, as a major eugregarine innovation. *PeerJ*, 9, e11912. https://doi.org/10.7717/peerj.11912

Pawlowski, J., Audic, S., Adl, S., Bass, D., Belbahri, L., Berney, C., Bowser, S.S., Cepicka, I., Decelle, J., Dunthorn, M., Fiore-Donno, A.M., Gile, G.H., Holzmann, M., Jahn, R., Jirků, M., Keeling, P.J., Kostka, M., Kudryavtsev, A., Lara, E., … de Vargas, C. (2012) CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. *PLoS Biology*, 10(11), e1001419. https://doi.org/10.1371/journal.pbio.1001419

Peakall, R. & Smouse, P.E. (2012) GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research –an update. *Bioinformatics*, 28, 2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Pedersen, A.B. & Fenton, A. (2007) Emphasizing the ecology in parasite community ecology. *Trends in Ecology & Evolution*, 22, 133–139. https://doi.org/10.1016/j.tree.2006.11.005

Petney, T.N. & Andrews, R.H. (1998) Multiparasite communities in animals and humans: frequency, structure and pathogenic significance. *International Journal for Parasitology*, 28, 377–393. https://doi.org/10.1016/s0020-7519(97)00189-6

Portanier, E., Garel, M., Devillard, S., Maillard, D., Poissant, J., Galan, M., Benabed, S., Poirel, M-T., Duhayer, J., Itty, C. & Bourgoin, G. 2019. Both candidate gene and neutral genetic diversity correlate with parasite resistance in female Mediterranean mouflon. *BMC Ecology,* 19:12. https://doi.org/10.1186/s12898-019-0228-x

Poulin, R. (1993) The disparity between observed and uniform distributions: a new look at parasite aggregation. *International Journal for Parasitology*, 23, 937–944. https://doi.org/10.1016/0020-7519(93)90060-C

Poulin, R. (2013) Explaining variability in parasite aggregation levels among host samples. *Parasitology*, 140, 541–546. https://doi.org/10.1017/S0031182012002053

Puurtinen, M., Hytönen, M., Knott, K.E., Taskinen, J., Nissinen, K. & Kaitala, V. (2004) The effects of mating system and genetic variability on susceptibility to trematode parasites in a freshwater snail, *Lymnaea stagnalis*. *Evolution*, 58, 2747–2753. https://doi.org/10.1111/j.0014-3820.2004.tb01626.x

R Core Team. (2021) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/

Read, A.F. & Taylor, L.H. (2001) The ecology of genetically diverse infections. *Science*, 11, 1099–1102. https://doi.org/10.1126/science.1059410

Rovenholt, F.H. & Tate, A.T. (2022) The impact of coinfection dynamics on host competition and coexistence. *The American Naturalist*, 199, 91–107. https://doi.org/10.1086/717180

Rueckert, S., Betts, E.L. & Tsaousis, A.D. (2019) The symbiotic spectrum: where do the gregarines fit? *Trends in Parasitology*, 35, 687–694. https://doi.org/10.1016/j.pt.2019.06.013

Saffo, M.B. (1982) Distribution of the endosymbiont *Nephromyces giard* within the ascidian family Molgulidae. *The Biological Bulletin*, 162, 95–104.

Saffo, M.B., McCoy, A.M., Rieken, C. & Slamovits, C.H. (2010) Nephromyces, a beneficial apicomplexan symbiont in marine animals. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 16190–16195. https://doi.org/10.1073/pnas.1002335107

Salomäki, E.D., Terpis, K.X., Rueckert, S., Kotyk, M., Varadínová, Z.K., Čepička, I. et al. (2021) Gregarine single-cell transcriptomics reveals differential mitochondrial remodeling and adaptation in apicomplexans. *BMC Biology*, 19, 77. https://doi.org/10.1186/s12915-021-01007-2

Seabloom, E.W., Borer, E., Gross, K., Kendig, A.E., Lacroix, C., Mitchell, C.E. et al. (2015) The community ecology of pathogens: coinfection, coexistence and community composition. *Ecology Letters*, 18, 401–415. https://doi.org/10.1111/ele.12418

Seeber, F. & Steinfelder, S. (2016) Recent advances in understanding apicomplexan parasites. *F1000Res.*, 5, F1000 faculty Rev-1369. https://doi.org/10.12688/f1000research.7924.1

Slate, J., Kruuk, L.E.B., Marshall, T.C., Pemberton, J.M. & Clutton-Brock, T.H. (2000) Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). *Proceedings of the Royal Society of London B*, 267, 1657–1662. https://doi.org/10.1098/rspb.2000.1192

Stevens, L., Yan, G. & Pray, L.A. (1997) Consequences of inbreeding on invertebrate host susceptibility to parasitic infection. *Evolution*, 51, 2032–2039. https://doi.org/10.2307/2411025

Sumner, R. (1936) Relation of gregarines to growth and longevity in the mealworm *Tenebrio molitor* L. *Annals of the Entomological Society of America*, 29, 645–648. https://doi.org/10.1093/aesa/29.4.645

Susi, H., Barrès, B., Vale, P.F. & Laine, A.-L. (2015) Co-infection alters population dynamics of infectious disease. *Nature Communications*, 6, 5975. https://doi.org/10.1038/ncomms6975

Thonig, A., Knott, K.E., Kesäniemi, J.E., Hansen, B.W. & Banta, G.T. (2016) Population and reproductive dynamics of the polychaete *Pygospio elegans* in a boreal estuary complex. *Invertebrate Biology*, 135, 370–384. https://doi.org/10.1111/ivb.1214

Thonig, A., Banta, G.T., Hansen, B.W. & Knott, K.E. (2017) Seasonal genetic variation associated with population dynamics of a poecilogonous polychaete worm. *Ecology and Evolution*, 7, 10005–10017. https://doi.org/10.1002/ece3.3518

Valigurová, A. (2012) Sophisticated adaptations of *Gregarina cuneata* (Apicomplexa) feeding stages for epicellular parasitism. *PLoS One*, 7, e42606. https://doi.org/10.1371/journal.pone.0042606

Velavan, T.P., Weller, S., Schulenburg, H. & Michiels, N.K. (2009) High genetic diversity and heterogeneous parasite load in the earthworm *Lumbricus terrestris* on a German meadow. *Soil Biology and Biochemistry*, 41, 1591–1595. https://doi.org/10.1016/j.soilbio.2009.03.026

Viney, M.E. & Graham, A.F. (2013) Chapter five – patterns and processes in parasite co-infection. In: Rollinson, D. (Ed.) *Advances in parasitology*, Vol. 82, 1st edition. Oxford: Academic Press, pp. 321–369. https://doi.org/10.1016/B978-0-12-407706-5.00005-8

Warwick, R.M. & Uncles, R.J. (1980) The distribution of benthic macrofauna associations in the Bristol Channel in relation to tidal stress. *Marine Ecology Progress Series*, 3, 97–103.

Webster, J.P. & Woolhouse, M.E.J. (1999) Cost of resistance: relationship between reduced fertility and increased resistance in a snail-schistosome host-parasite system. *Proceedings of the Royal Society of London B*, 266, 391–396. https://doi.org/10.1098/rspb.1999.0650

Weir, B.S. & Cockerham, C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358–1370.

Whitehorn, P.R., Tinsley, M.C., Brown, M.J., Darvill, B. & Goulson, D. (2011) Genetic diversity, parasite prevalence and immunity in wild bumblebees. *Proceedings of the Royal Society B*, 278, 1195–1202. https://doi.org/10.1098/rspb.2010.1550

Zélé, F., Nicot, A., Berthomieu, A., Weill, M., Duron, O. & Rivero, A. (2014) *Wolbachia* increases susceptibility to *plasmodium* infection in a natural system. *Proceedings of the Royal Society B*, 281, 20132837. https://doi.org/10.1098/rspb.2013.2837

Zélé, F., Magalhães, S., Kéfi, S. & Duncan, A.B. (2018) Ecology and evolution of facilitation among symbionts. *Nature Communications*, 9, 4869. https://doi.org/10.1038/s41467-018-06779-w

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hiillos, A-L, Rony, I., Rueckert, S. & Knott, K.E. (2023) Coinfection patterns of two marine apicomplexans are not associated with genetic diversity of their polychaete host. *Journal of Eukaryotic Microbiology*, 70, e12932. Available from: https://doi.org/10.1111/jeu.12932