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Two parasites in one host: spatiotemporal dynamics and co-occurrence of Microsporidia and *Rickettsia* in an amphipod host

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Research Article

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Abstract

Biological interactions can greatly influence the abundance of species. This is also true for parasitic species that share the same host. Microsporidia and *Rickettsia* are widespread intracellular parasites in populations of *Paracalliope* fluviatilis, the most common freshwater amphipods in New Zealand. Although both parasites coexist in many populations, it is unclear whether they interact with each other. Here, we investigated spatial–temporal dynamics and co-occurrence of the two parasites, Microsporidia and *Rickettsia* in *P. fluviatilis* hosts, across one annual cycle and in three different locations. Prevalence of both Microsporidia and *Rickettsia* changed over time. However, while the prevalence of *Rickettsia* varied significantly between sampling times, that of Microsporidia did not change significantly and remained relatively low. The two parasites therefore followed different temporal patterns. Also, the prevalence of both parasites differed among locations, though the two species reached their highest prevalence in different locations. Lastly, there was no evidence for positive or negative associations between the two parasite species; the presence of one parasite in an individual host does not appear to influence the probability of infection by the other parasite. Their respective prevalence may follow different patterns among populations on a larger spatial scale due to environmental heterogeneity across locations.

Introduction

The abundance of any species in any habitat varies over time. Environmental factors such as temperature and precipitation drive abundance in many organisms (Pollard *et al.*, 1999; White *et al.*, 2000), and so do biological interactions (Woodin, 1974; Martins and Haimovici, 1997). For example, the density of prey and predators in the habitat (Arditi and Ginzburg, 1989), competition for resources (Robertson, 1996) and parasites and diseases can all contribute to shape the abundance and dynamics of animal populations (Scott and Dobson, 1989; Poulin, 1999). The prevalence of parasites themselves is also governed by various factors. During free-living stages, the prevalence of parasites can be largely influenced by abiotic factors similar to other free-living organisms (Altizer *et al.*, 2006). In addition, because parasites are highly dependent on their host for survival at least for part of their life cycle, all environmental and biological factors that influence host abundance can, directly and indirectly, also affect the prevalence of their parasites (Arneberg *et al.*, 1998). More specifically, changes in host behaviour, host immune response and fluctuating host births and deaths (i.e. changes in host density) themselves can alter the prevalence of parasites in a host population (Grassly and Fraser, 2006).

Another important factor that determines the prevalence of parasites is the interaction among parasites that use the same hosts; depending on their mode of transmission, virulence and their ability to manipulate the hosts, various outcomes are possible (Haine *et al.*, 2005; Rigaud *et al.*, 2010; Poulin, 2011). For example, vertically transmitted parasites (= parasites that are transmitted from a mother to offspring) often have little or no effect on their host's fitness because the hosts' reproductive success is crucial for the parasite as well (Dunn and Smith, 2001). On the other hand, horizontally transmitted parasites (= parasites that are transmitted between different individuals) are generally involved with severe pathology (Dunn and Smith, 2001). Some trophically (= horizontally) transmitted parasites are capable of changing host behaviour, which ultimately leads to predation of the intermediate host by the final host (Thomas and Poulin, 1998). Parasites with these contrasting modes of transmission sharing the same host are therefore in conflict. For example, in an amphipod host, the coinfection of vertically transmitted microsporidia has been shown to weaken the behavioural alteration induced by trophically transmitted acanthocephalans (Haine *et al.*, 2005). In contrast, parasites with the same transmission mode that share the same host may have interests that are well aligned. For instance, in an amphipod host, two vertically transmitted parasites, one microsporidian and one paramyxean, were shown to co-occur more frequently than expected by chance; since feminization of male hosts is necessary for successful vertical transmission of the parasites, this suggested that one parasite was hitchhiking with another capable of feminizing the host (Short *et al.*, 2012), or that both can feminize their hosts (Arundell *et al.*, 2015; Pickup and Ironside, 2018).

Amphipods host diverse macroparasites (trematodes, acanthocephalans and nematodes) as well as diverse microparasites (viruses, bacteria and protists) (Bethel and Holmes, 1977; Poulin and Latham, 2002; Bojko and Ovcharenko, 2019; Friesen *et al*., 2019). Amphipods are used as intermediate hosts by many parasites (Dezfuli *et al*., 2000), and a single amphipod individual can be infected by several different groups of parasites at the same time (Haine *et al*., 2005; Short *et al*., 2012). Recently, some lineages of microsporidians including three dictyocoelan species (*Dictyocoela* sp. NZ1-3), as well as Torix group of *Rickettsia* were shown to be widespread in multiple New Zealand populations of *Paracalliope fluviatilis* amphipods (Park and Poulin, 2020; Park *et al*., 2020). *Dictyocoela* is so far the most common microsporidian genus in amphipod hosts in both Northern and Southern Hemispheres and at least 17 species-level taxa are known (Bacela-Spychalska *et al*., 2018; Park *et al*., 2020). While horizontal transmission is the most common mode of transmission among microsporidians, vertical transmission is also known to occur in multiple species of *Dictyocoela* (Terry *et al*., 2004). It is believed that *Dictyocoela* can be transmitted mainly vertically, horizontally, or both. However, which is the primary mode of transmission in each species is poorly understood (Quiles *et al*., 2020). Torix *Rickettsia* are known to be common in aquatic invertebrate hosts but their presence in amphipod hosts was only recently documented (Park and Poulin, 2020). *Rickettsia* in general are believed to be transmitted vertically (Weinert *et al*., 2009), and strong evidence for vertical transmission (the presence of *Rickettsia* in the oocytes and developing embryos) has been reported in some insects and leeches (Kikuchi and Fukatsu, 2005; Küchler *et al*., 2009; Pilgrim *et al*., 2017). However, basic biology including the mode of transmission of these recently discovered microsporidians and *Rickettsia* in New Zealand amphipod hosts remains largely unknown.

The two parasites often coexist in the same host population, however, whether they interact with each other is unknown. If they are both vertically transmitted, they may compete for space within the same individual host (e.g. within gonadal cells to be transmitted to offspring *via* gametes), which could lead to the prior infection by one parasite causing the exclusion of the other parasite from the same individual host. On the other hand, they may also be positively associated. For example, since some microsporidians can feminize their amphipod host (Dunn *et al*., 2001) and therefore could improve their chances of transmission to the next generation (vertical transmission is only possible from female hosts to their offspring), *Rickettsia* may benefit from associating with microsporidian-infected host individuals. The question is then, do they tend to coinfect the same individual amphipod more frequently than expected by chance, avoid each other, or are there no associations between them?

Both microsporidians and *Rickettsia* include species of economic importance and serious pathogens in humans, livestock and companion animals, and the seasonal dynamics of these pathogens have received more attention. For example, microsporidian keratitis peaks during the rainy season in several countries (Reddy *et al*., 2011; Tham and Sanjay, 2012). Rocky Mountain spotted fever caused by *Rickettsia rickettsia* peaks during seasons when vector species (i.e. ticks) are abundant (Walker, 1995). However, the prevalence of parasites follows different temporal trends in different host systems. A recent meta-analysis showed that there is no universal pattern in the seasonal dynamics of aquatic metazoan parasites; instead seasonal variation in infection levels depends on taxa and habitat (Poulin, 2020). Microsporidians also show various temporal trends driven by different factors. For example, *Octosporea bayeri* in *Daphnia* hosts showed clear cyclic prevalence patterns increasing in summer and decreasing in winter. This was related to the host lifecycle (i.e. diapause) rather than external temperature (Lass and Ebert, 2006). Other microsporidians, also in *Daphnia* but in different locations, showed more-or-less constant prevalence over space and time (Wolinska *et al*., 2011). Microsporidian species in *Artemia* displayed a clear pattern of seasonality although this was affected by the presence of other host species (Lievens *et al*., 2019). Therefore, different host–parasite associations can be characterized by various patterns of temporal prevalence fluctuations.

Here, we investigate patterns of temporal variations in prevalence and in the co-occurrence of Microsporidia and *Rickettsia* in *P. fluviatilis* host individuals and populations. We ask several specific questions: Does the prevalence of Microsporidia and *Rickettsia* change throughout the year? If so, do they have similar temporal patterns in different locations? Are their temporal variations in prevalence associated with host population dynamics? Do microsporidians and *Rickettsia* tend to co-infect the same individual hosts, or not? In order to answer these questions, we sampled *P. fluviatilis* specimens across an entire annual cycle from three different locations. We use molecular tools to quantify seasonal infection dynamics of both parasites as well as seasonal changes in host demographic parameters, and we test whether the co-occurrence of the two parasites among individual hosts departs from random.

Materials and methods

Field sampling

Three sampling sites on the South Island of New Zealand (S34, S37, S40; Fig. 1) were chosen among sites with both Microsporidia and *Rickettsia* based on the screening results from previous studies (Park and Poulin, 2020; Park *et al*., 2020). These sites were visited every two months between February 2019 and February 2020 (a total of seven sampling times). Individuals of *P. fluviatilis* were collected with dipnets and fine sieve nets; samples were collected among littoral macrophytes, in a standardized manner across localities and sampling times. Samples were stored in containers with 96% ethanol upon collection and then brought to the lab.

Sample preparation

For each population and for each sampling time, 24 individuals were randomly chosen for molecular screening for parasite detection. They were sexed under a microscope and then were photographed using a DP25 camera mounted on a microscope and the Olympus DP2-BSW application software. These photos were later used to measure the body size of each amphipod individual. The distance from the base of the first antennal segment to the base of the telson (Asochakov, 1994) was recorded as a measure of body size using ImageJ software (http://imagej.nih.gov/ij/). The brood size (= number of eggs in a brood pouch) was recorded for each mature female. After being washed with distilled water, the whole body was used for DNA extraction for each individual amphipod.

Parasite detection by PCR

The presence of Microsporidia and *Rickettsia* for each amphipod individual was detected by PCR, For Microsporidia, a primer pair of V1f (CACCAGGTTGATTCTGCCTGAC) and MC3R (GATAACGA CGGGCGGTGTGTACAA) targeting a partial small ribosomal RNA region were used (Zhu *et al*., 1993; Ovcharenko *et al*., 2010). For *Rickettsia*, a primer pair of Ri170_F (GGGCTTGCTCTAAATTAGTTAGT) and Ri1500_R (ACGTTAGCTCACCACCTTCAGG) also for a partial small ribosomal RNA region (Küchler *et al*., 2009). PCR reactions were

Fig. 1. Map of New Zealand's South Island showing sampling locations. *Paracalliope fluviatilis* specimens were collected from the three sites in the Otago and Southland regions within one-year span.

conducted following conditions described in Park *et al.* (2020) and Park and Poulin (2020) for microsporidians and *Rickettsia*, respectively. For each set of PCR reactions, both negative and positive controls were included. 1–2 PCR products per population were sent to Genetic Analysis Services at the University of Otago, New Zealand for sequencing, and all were confirmed as *Dictyocoela* spp. (the most dominant microsporidian group in amphipod hosts) for Microsporidia and Torix group for *Rickettsia*.

Data analysis

The temporal fluctuations in the prevalence of each parasite and their co-occurrence in three different locations were visually represented as stacked bar graphs (Fig. 2A), whereas temporal variations in amphipod body sizes (males and females separately) and the brood size of females were plotted as boxplots (Fig. 2B–D). All plots were generated using the *ggplots2* package (Wickham, 2011) in R environment (version 3.5.2; R Core Team, 2021).

We used two generalized linear models (GLM), one for microsporidians and one for *Rickettsia*, to evaluate the influence of several factors on the occurrence of each parasite in individual amphipods. The presence of the parasite was used as response variable (binomial distribution: uninfected = 0, infected = 1). We assessed several fixed factors: presence of the other parasite in the amphipod (absent = 0, present = 1), sampling time, location, sex and amphipod body size for their effects on the focal parasite's occurrence. 'Sampling time' had seven levels (19 Feb, 19 Apr, 19 Jun, 19 Aug, 19 Oct, 19 Dec and 20 Feb), 'location' had three levels (S34, S37 and S40) and 'sex' had two levels (female and male). None of the data was transformed. The GLMs were conducted using '*glm*' function in the '*lme4*' package (Bates *et al.*, 2015). Pairwise comparisons across different 'locations' and 'sampling times' were performed using the *glht* function in the *multcomp* package (Hothorn *et al.*, 2008). All statistical analyses were conducted in the R environment (version 3.5.2; R Core Team, 2021).

Results

Host population dynamics

P. fluviatilis populations persisted throughout the year in all three locations (Fig. 1), although we failed to collect specimens in the S34 population from one sampling time (19 Dec). Amphipod demographic parameters, i.e. body size and brood size, showed clear temporal variations in all locations (Fig. 2B–D). It seems that *P. fluviatilis* is most productive during the austral spring (September–November), based on our observation of the highest brood size in females from the October samples, across all three populations. Brood sizes appear to decrease during the summer and early winter (December–June). In June, females harbouring eggs were very rare in all locations (Fig. 2B). The body size of the females and males also varied greatly throughout the year, showing similar patterns with that of brood size. It seems that mature females and males are mostly found during the late winter to spring (August–October). During autumn to winter (February–June), the populations consisted mostly of small, immature individuals (Fig. 2C–D).

Spatiotemporal variations in parasite prevalence

Both Microsporidia and *Rickettsia* were found from all three locations, but their prevalence changed throughout the year (Fig. 2A). *Rickettsia* was found in all locations at all sampling times, but Microsporidia were not found at some sampling times. The prevalence of *Rickettsia* showed ranges of 8.3–37.5%, 16.7–95.8% and 4.2–41.7% in the S34, S37 and S40 populations, respectively. On the other hand, the prevalence of Microsporidia was generally low, i.e. 0–8.3% and 0–12.5% in the S37 and S40 populations, respectively, although its prevalence reached up to 25% in winter at the S34 population. The GLM results supported the lack of temporal variation in the prevalence of Microsporidia (Table 1). On the other hand, the effect of sampling time (temporal effect) on *Rickettsia* infections was supported by GLM results (Table 2). Compared to the first sampling time (February), the April samples showed a significantly lower prevalence of *Rickettsia*. Also, locations had effects on the prevalence of both Microsporidia and *Rickettsia*, indicating that different host populations tend to have a different prevalence of both parasites. The S37 population has the highest prevalence of *Rickettsia* ($z = 8.23$, $P < 2 \times 10^{-16}$). The pairwise comparisons of the effects of different sampling times and locations, respectively, on infections by each parasite, are shown in Tables 3 and 4.

Co-occurrence of the two parasites

Individuals simultaneously harbouring both Microsporidia and *Rickettsia* were found in all locations and from several different sampling times (Fig. 2A). According to the GLM results, there was no effect of the infection by one parasite on the presence or absence of the other parasite (Tables 1 and 2). In other words, the presence of Microsporidia did not predict the presence of *Rickettsia* at the individual host level ($z = 0.10$, $P = 0.92$), and vice versa ($z = 0.22$, $P = 0.82$).

Discussion

We investigated the spatiotemporal variations in the prevalence of two parasites, Microsporidia and *Rickettsia*, sharing the same *P. fluviatilis* amphipod host species. There was no clear temporal variation in the prevalence of Microsporidia, but *Rickettsia* showed some temporal variations in prevalence among different sampling times. The prevalence of both parasites varied across locations, however, the patterns were different: Microsporidia were more common in the S34 population, and *Rickettsia* was more common in the S37 and S40 populations (Fig. 2 and Tables 1–4). Here, we discuss whether the observed temporal variation in the prevalence of *Rickettsia* may be associated with host population dynamics. Then, we discuss various factors that influence the temporal infection patterns within a population as well as spatial patterns at a larger scale. We also discuss the possible causes for the lack of co-occurrence of the two parasites at the individual host level.

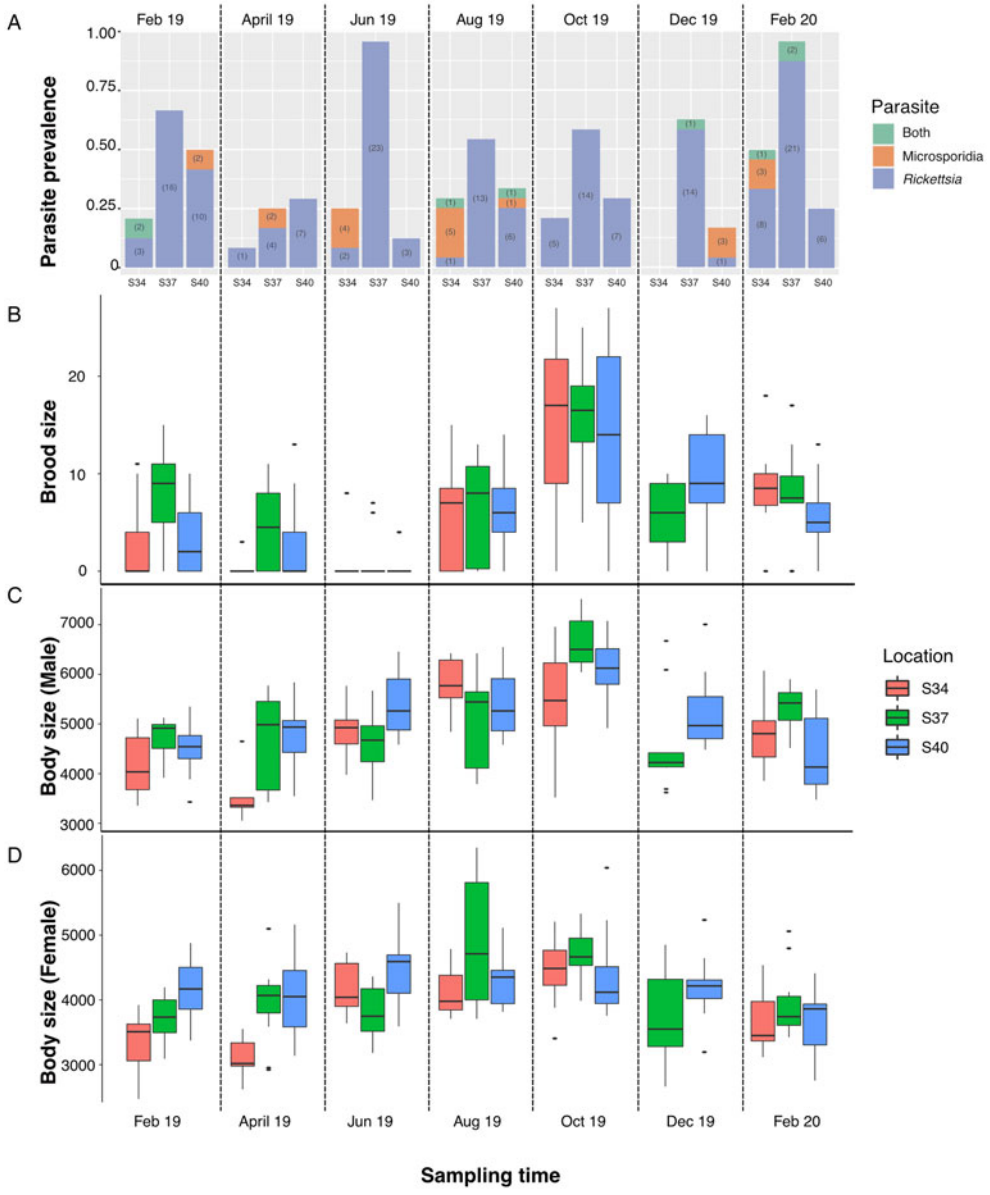

Fig. 2. The temporal variations of (A) parasite prevalence in three locations, (B) and the brood size (=number of eggs) of amphipod females, the body size (μm) of (C) males and (D) females. For box plots, medians (central lines), 25–75 percentiles (boxes), non-outliers (whiskers) and outliers (dots) are shown. All these traits are aligned together (with dashed lines) for better comparison of the temporal trends.

Table 1. Glm result showing the effects of various factors on Microsporidia infections in *Paracalliope fluviatilis* amphipods. *P* values less than 0.05 are highlighted in bold.

Fixed effects	Estimate	Standard error	Z-value	*P* value
(Intercept)	-1.11×10^{0}	1.30×10^{0}	−0.854	0.39335
Rickettsia	1.16×10^{-1}	5.22×10^{-1}	0.223	0.82365
body_size	-2.02×10^{-4}	3.46×10^{-4}	−0.584	0.55916
Sampling2_April-19	-2.95×10^{-1}	9.11×10^{-1}	−0.323	0.74636
Sampling3_Jun-19	2.56×10^{-1}	7.75×10^{-1}	0.33	0.74109
Sampling4_Aug-19	1.03×10^{0}	7.35×10^{-1}	1.396	0.16278
Sampling5_Oct-19	-1.65×10^{1}	1.20×10^{3}	−0.014	0.98904
Sampling6_Dec-19	1.36×10^{0}	8.23×10^{-1}	1.65	0.09896
Sampling7_Feb-20	5.13×10^{-1}	6.95×10^{-1}	0.738	0.46066
LocationS37	-1.87×10^{0}	6.60×10^{-1}	−2.83	**0.00466**
LocationS40	-1.42×10^{0}	5.31×10^{-1}	−2.672	**0.00754**
sexM	-4.72×10^{-1}	5.36×10^{-1}	−0.88	0.37899

Table 2. Glm result showing the effects of various factors on *Rickettsia* infections in *Paracalliope fluviatilis* amphipods. *P* values less than 0.05 are highlighted in bold.

Fixed effects	Estimate	Standard error	*Z*-value	*P* value
Intercept	0.0653594	0.676144	0.097	0.92299
Microsporidia	0.0511338	0.4990964	0.102	0.9184
body_size	−0.0003432	0.0001677	−2.047	**0.04062**
Sampling2_April-19	−1.5958317	0.4540489	−3.515	**0.00044**
Sampling3_Jun-19	−0.0306738	0.3996621	−0.077	0.93882
Sampling4_Aug-19	−0.4487632	0.4228839	−1.061	0.2886
Sampling5_Oct-19	−0.081639	0.4364869	−0.187	0.85163
Sampling6_Dec-19	−1.0378586	0.4504977	−2.304	**0.02123**
Sampling7_Feb-20	0.55359	0.3826242	1.447	0.14795
LocationS37	2.6256841	0.3192275	8.225	**$<2 \times 10^{-16}$**
LocationS40	0.7101636	0.312914	2.27	**0.02324**
sexM	−0.1995707	0.2717154	−0.734	0.46265

Table 3. The pairwise comparisons of the effects between 'sampling time' 'location' on Microsporidia infections in *Paracalliope fluviatilis* amphipods from the GLM results. *P* values less than 0.05 are highlighted in bold.

Fixed effects	Estimate	Standard error	*Z*-value	*P* value
Sampling time				
2_April-19–1_Feb-19 == 0	−0.2946	0.9109	−0.323	1
3_Jun-19–1_Feb-19 == 0	0.2562	0.7754	0.33	1
4_Aug-19–1_Feb-19 == 0	1.0264	0.7354	1.396	0.77
5_Oct-19–1_Feb-19 == 0	−16.4709	1198.8638	−0.014	1
6_Dec-19–1_Feb-19 == 0	1.3585	0.8234	1.65	0.603
7_Feb-20–1_Feb-19 == 0	0.5125	0.6947	0.738	0.987
3_Jun-19–2_April-19 == 0	0.5508	0.9365	0.588	0.996
4_Aug-19–2_April-19 == 0	1.3211	0.9062	1.458	0.732
5_Oct-19–2_April-19 == 0	−16.1763	1198.864	−0.013	1
6_Dec-19–2_April-19 == 0	1.6532	0.9509	1.738	0.541
7_Feb-20–2_April-19 == 0	0.8071	0.8801	0.917	0.962
4_Aug-19–3_Jun-19 == 0	0.7702	0.6696	1.15	0.893
5_Oct-19–3_Jun-19 == 0	−16.7271	1198.8638	−0.014	1
6_Dec-19–3_Jun-19 == 0	1.1024	0.8054	1.369	0.786
7_Feb-20–3_Jun-19 == 0	0.2563	0.7072	0.362	1
5_Oct-19–4_Aug-19 == 0	−17.4973	1198.8637	−0.015	1
6_Dec-19–4_Aug-19 == 0	0.3321	0.7405	0.449	0.999
7_Feb-20–4_Aug-19 == 0	−0.5139	0.6482	−0.793	0.982
6_Dec-19–5_Oct-19 == 0	17.8294	1198.8639	0.015	1
7_Feb-20–5_Oct-19 == 0	16.9834	1198.8638	0.014	1
7_Feb-20–6_Dec-19 == 0	−0.846	0.7646	−1.106	0.909
Location				
S37 – S34 == 0	−1.8676	0.66	−2.83	**0.0128**
S40 – S34 == 0	−1.4181	0.5307	−2.672	**0.0203**
S40 – S37 == 0	0.4495	0.667	0.674	0.7772

Although clear temporal variations in the prevalence of *Rickettsia* along with seasonal changes were not seen, the April samples showed low prevalence in all populations compared to that of the previous sampling time (i.e. February). The difference in the prevalence of *Rickettsia* between the April and February samples is also supported by the pairwise comparison of means (Table 2). Because we only have data for one year, we do not know if the same patterns are consistent across years. The decrease

Table 4. Pairwise comparisons of the effects between 'sampling time' 'location' on *Rickettsia* infections in *Paracalliope fluviatilis* amphipods from the GLM results. *P* values less than 0.05 are highlighted in bold.

Fixed effects	Estimate	Standard error	*Z*-value	*P* value
Sampling time				
2_April-19–1_Feb-19 == 0	−1.59583	0.45405	−3.515	**0.00782**
3_Jun-19–1_Feb-19 == 0	−0.03067	0.39966	−0.077	1
4_Aug-19–1_Feb-19 == 0	−0.44876	0.42288	−1.061	0.93845
5_Oct-19–1_Feb-19 == 0	−0.08164	0.43649	−0.187	1
6_Dec-19–1_Feb-19 == 0	−1.03786	0.4505	−2.304	0.2397
7_Feb-20–1_Feb-19 == 0	0.55359	0.38262	1.447	0.77391
3_Jun-19–2_April-19 == 0	1.56516	0.46448	3.37	**0.01313**
4_Aug-19–2_April-19 == 0	1.14707	0.47767	2.401	0.19558
5_Oct-19–2_April-19 == 0	1.51419	0.4933	3.07	**0.03461**
6_Dec-19–2_April-19 == 0	0.55797	0.49267	1.133	0.91716
7_Feb-20–2_April-19 == 0	2.14942	0.456	4.714	**< 0.001**
4_Aug-19–3_Jun-19 == 0	−0.41809	0.41669	−1.003	0.95276
5_Oct-19–3_Jun-19 == 0	−0.05097	0.42184	−0.121	1
6_Dec-19–3_Jun-19 == 0	−1.00718	0.45974	−2.191	0.29772
7_Feb-20–3_Jun-19 == 0	0.58426	0.39391	1.483	0.75262
5_Oct-19–4_Aug-19 == 0	0.36712	0.4144	0.886	0.97438
6_Dec-19–4_Aug-19 == 0	−0.5891	0.47154	−1.249	0.87313
7_Feb-20–4_Aug-19 == 0	1.00235	0.41535	2.413	0.19126
6_Dec-19–5_Oct-19 == 0	−0.95622	0.48791	−1.96	0.43737
7_Feb-20–5_Oct-19 == 0	0.63523	0.42539	1.493	0.74664
7_Feb-20–6_Dec-19 == 0	1.59145	0.45235	3.518	**0.00791**
Location				
S37 – S34 == 0	2.6257	0.3192	8.225	**$<1\times10^{-04}$**
S40 – S34 == 0	0.7102	0.3129	2.27	0.0595
S40 – S37 == 0	−1.9155	0.2624	−7.3	**$<1\times10^{-04}$**

in the prevalence in April could have been due to seasonal environmental change, stochastic processes, or it may be related to the change of the amphipod cohort. It may also be the product of the relatively modest sample sizes used in the analyses. The body size of both females and males was lowest during April, which means that the previous cohorts probably died and most individuals in the population at that time were a new, young cohort. As an obligate parasite, the strong dependence of *Rickettsia* on the host is assumed (Sibley, 2004), but more long-term data will be needed to better understand if there are indeed seasonal fluctuations in infection prevalence, and if so, what factors drive those patterns.

Host–parasite associations are shaped by various biotic and abiotic factors (Anderson and Sukhdeo, 2010). These factors are not the same among localities. The compositions of potential host and parasite species differ among localities, as do climatic and other environmental factors. Therefore, the absence of general temporal patterns may be due to biotic and abiotic heterogeneity among habitats. Although we did not find any evidence of non-random coinfection (or avoidance) patterns between Microsporidia and *Rickettsia* at the individual level in our three study sites, it is still possible that their distributions on a larger spatial scale are not mutually independent. Indeed, there is a difference in the spatial distribution of the two parasites across New Zealand. Although Microsporidia have been found throughout the country, *Rickettsia* was found only in the southern part of North Island and the southern part of the South Island (Park and Poulin, 2020; Park *et al.*, 2020). The reason for the absence of *Rickettsia* in the northern parts of both Islands is not understood yet but could be due to phylogeographic or environmental processes; this remains to be studied.

If and how microsporidians and *Rickettsia* interact with each other within the same host individual is not understood, but their interaction and co-occurrence can be largely shaped by their mode of transmission, and this could affect our interpretation. For example, even if both microsporidians and *Rickettsia* infect the same individual, the exact host organs and tissues they target (i.e. tissue tropism) and how they interact with the host cell may be different (Sahni and Rydkina, 2009; Tamim El Jarkass and Reinke, 2020). If one is transmitted horizontally and the other is transmitted vertically, they are likely to be located in different tissues and organs and might not need to compete for the same resources. For instance, horizontally transmitted parasites are often found in the gut epithelium. On the other hand, vertically transmitted parasites are found from gonadal tissues (Dunn *et al.*, 2001). If both parasites are mainly transmitted vertically, they may cause little or no pathogenic effects on the host and there would be no obvious conflict between them (Rigaud *et al.*, 2010). Intracellular endosymbionts have generally shown low virulence to their hosts (Dunn *et al.*, 2001), therefore they may be able to coexist in the same individual host without strong conflicts. Elucidating mode of transmission in these newly found parasites would be needed to better understand their interactions.

Although evidence for strong conflicts between microsporidians and *Rickettsia* has not been seen, these endosymbionts may have conflicts of interests with other horizontally (including trophically) transmitted parasites. *P. fluviatilis* is an important prey item for fishes in New Zealand and is used as intermediate host by several different helminth parasites including trematodes and acanthocephalans (Lagrue *et al*., 2016; Friesen *et al*., 2019). While dissecting amphipods, we found a trematode species, *Coitocaecum parvum* with varying prevalence across sampling times and locations, but they did not show any positive or negative co-occurrence patterns with Microsporidia or *Rickettsia* (data not shown). However, still, complex interactions, on both ecological and evolutionary time scales, may exist between Microsporidia, *Rickettsia* and other macro- and microparasites, and these may be involved in generating observed patterns in the spatial distribution of the two focal parasites in this study. There are other factors that could also influence the co-occurrence patterns (e.g. demographic structure of hosts and uneven distribution of parasites within a habitat). More information about host and parasite biology would be valuable to disentangle various factors that shape complex interactions among them.

It also should be noted that different species or different strains of the same species of the parasites may have different pathogenicity and interact with other parasites and hosts differently. Among microsporidians, six species of *Dictyocoela* were detected in New Zealand so far, and three of them (*D*. sp. NZ1-3) were exclusively found from *Paracalliope* spp. showing evidence for host fidelity (Park *et al*., 2020). In this study, *D*. sp. NZ2 was detected in the S37 population and D. sp. NZ3 was detected from the S34 and S40 populations. Also, several strains of *Rickettsia* were detected, but if they have different pathogenicity has not been shown yet (Park and Poulin, 2020).

From an evolutionary perspective, the coexistence of Microsporidia and *Rickettsia* in amphipod hosts is interesting. Canonical Microsporidia have several unique characteristics that make them different from their relatives (Park and Poulin, 2021). One of the unique characteristics is the presence of ADP/ATP translocators (Dean *et al*., 2016), which allows microsporidians to effectively 'steal' energy from the host cell. These translocators are highly similar to those of *Rickettsia* or *Chlamydia*, and therefore it is believed that these translocators were horizontally obtained from these bacteria or their ancestors (Dean *et al*., 2016). Physical proximity must be a requirement for horizontal gene transfer. Therefore, it is likely that the ancestors of long-branch microsporidia and *Rickettsia* shared the same host cell. Their coexistence within the same host cell may have provided the evolutionary novelty which has led their joint evolutionary success.

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