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Long-term prevalence data reveals spillover dynamics in a multi-host (*Artemia*), multi-parasite (Microsporidia) community

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ABSTRACT

In the study of multi-host parasites, it is often found that host species contribute asymmetrically to parasite transmission. Yet in natural populations, identifying which hosts contribute to parasite transmission and maintenance is a recurring challenge. Here, we approach this issue by taking advantage of natural variation in the composition of a host community. We studied the brine shrimps *Artemia franciscana* and *Artemia parthenogenetica* and their microsporidian parasites *Anostracospora rigaudi* and *Enterocytospora artemiae*. Previous laboratory experiments had shown that each host can transmit both parasites, but could not predict their actual contributions to the parasites' maintenance in the field. To resolve this, we gathered long-term prevalence data from a metacommunity of these species. Metacommunity patches could contain either or both of the *Artemia* host species, so that the presence of the hosts could be linked directly to the persistence of the parasites. First, we show that the microsporidian *A. rigaudi* is a spillover parasite: it was unable to persist in the absence of its maintenance host *A. parthenogenetica*. This result was particularly striking, as *A. rigaudi* displayed both high prevalence (in the field) and high infectivity (when tested in the laboratory) in both hosts. Moreover, the seasonal presence of *A. parthenogenetica* imposed seasonality on the rate of spillover, causing cyclical pseudo-endemics in the spillover host *A. franciscana*. Second, while our prevalence data was sufficient to identify *E. artemiae* as either a spillover or a facultative multi-host parasite, we could not distinguish between the two possibilities. This study supports the importance of studying the community context of multi-host parasites, and demonstrates that in appropriate multi-host systems, sampling across a range of conditions and host communities can lead to clear conclusions about the drivers of parasite persistence.

1. Introduction

Although many parasites infect multiple host species within their community (Cleaveland et al., 2001; Taylor et al., 2001), not all hosts are of equal importance. Host species vary widely in their degree of exposure to parasites (e.g. Kilpatrick et al., 2006), competence (e.g. LoGiudice et al., 2003; Auld et al., 2017), and population density (e.g. Dobson, 1995; Rhodes et al., 1998; Searle et al., 2016). These factors affect their contribution to the parasite's overall transmission, and thus to the maintenance of a persistent parasite population (Dobson, 2004; Streicker et al., 2013).

Quantifying the relative contribution of each host species to the persistence of a multi-host parasite is important for several reasons. Host species that contribute substantially to the parasite's maintenance have a strong influence on its epidemiology (Viana et al., 2014) and evolutionary trajectory (Holt and Hochberg, 2002; Benmayor et al., 2009; Ching et al., 2013), and can be pinpointed in the development of disease control strategies (Fenton and Pedersen, 2005; Streicker et al., 2013). Conversely, host species that do not contribute much to transmission can act as ecological sinks, weakening the parasite's foothold in the community ("diluting" it, see Ostfeld and Keesing, 2000; Holt et al., 2003). Correctly identifying where hosts fall on this spectrum is therefore an essential prerequisite to understanding the epidemiological dynamics and evolution of any multi-host parasite. Furthermore, identifying the contributions of different host species may reveal unexpected community dynamics, as asymmetrical transmission of parasites between host species can feed back into the broader community structure (host species and their prey, competitors, and predators; reviewed in Hatcher et al., 2006).

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Identifying which host species contribute to the persistence of a natural parasite population is notoriously difficult (Viana et al., 2014). Parasites can persist in a host population if their basic reproduction number R_0 is greater than one (Kermack and McKendrick, 1927); the host is then said to maintain the parasite population. In multi-host communities, each host species contributes to the parasite's overall R_0 (Dobson, 2004), and an R_0 greater than one can mask a lot of variation in those contributions. For example, Fenton et al. (2015) considered a parasite infecting a simple two-host community, finding that it may fall into three underlying categories: i) a facultative multi-host parasite, which can be maintained by either host in the absence of the other; ii) an obligate multi-host parasite, which can only persist when both hosts are present; iii) a spillover parasite, which can be maintained indefinitely by one of the host species, but not by the other. In the final case, the parasite's presence in the second, 'spillover', host is dependent on regular reintroductions from the 'reservoir' or 'maintenance' host (Ashford, 1997; Haydon et al., 2002). Distinguishing between these fundamentally distinct, but superficially similar, categories is challenging. In the specific case of 'dead-end' hosts that cannot transmit the parasite, a simple experiment can prove that there is a spillover. For all other multi-host parasites, laboratory-based tests of host competence are insufficient: parasite traits can be very different under field conditions (e.g. virulence and growth, Bedhomme et al., 2004; Dunn et al., 2006), and host densities are key (Searle et al., 2016). Solutions include the establishment of epidemiological, statistical, or genetic models, which may be labor-intensive and sensitive to assumptions (Viana et al., 2014).

Another way to identify into which category a multi-host parasite falls is to exploit variation in the composition of natural host communities (Fig. 1). For example, a parasite which can be found in communities containing both hosts A and B, but also in isolated populations of host A or host B, is clearly a facultative multi-host parasite. In contrast, a parasite that spills over from host A to host B can be identified by its presence in communities containing host A or hosts A and B, but repeated absence in communities containing only host B. This type of observation explicitly links the presence or absence of hosts to the presence or absence of disease, and can therefore lead to direct conclusions. Previously, such conclusions have mostly been drawn from human interventions such as the vaccination or culling of a suspected reservoir (e.g. Dobson, 1995; Caley et al., 1999; MacInnes et al., 2001; Nugent, G., 2005. The role of wild deer in the epidemiology and management of bovine tuberculosis in New Zealand. PhD Thesis, Lincoln University, New Zealand; Serrano et al., 2011), or through the experimental construction of host communities (e.g. Power and Mitchell, 2004; Searle et al., 2016). However, the approach need not be limited to created variation. We can also look for 'natural experiments', host communities whose composition varies in the field. Of course, as natural experiments are not planned, due caution must be taken with regard to potential confounding factors. For instance, the epidemiology of a focal parasite may be shaped by an environmental variable (e.g. temperature, Altizer et al., 2006; Dunn et al., 2006), which happens to covary with the presence of a certain host. Stochastic effects may also play a larger role in natural experiments. Even in a suitable host community (gray squares in Fig. 1), the establishment of a parasite may be prevented by random environmental changes. To ensure that the conclusions are robust to any such effects, the observations must be repeated many times and across a range of relevant field conditions (e.g. different temperatures).

We illustrate the natural experiment approach using a two-host, two-parasite system: the brine shrimps *Artemia franciscana* and *Artemia parthenogenetica*, and their microsporidian parasites *Anostracospora rigaudi* and *Enterocytospora artemiae*. The hosts and parasites occur in sympatry in southern France, where both microsporidians can be found on both hosts (Rode et al., 2013a,b). Laboratory experiments have shown that the two microsporidians are partially specialized. Each host is more competent for one of the two parasites, but can transmit both (Lievens et al., 2018). Thus neither host is a dead-end, but their contribution to the parasites' maintenance in the field could be small or substantial – we cannot extrapolate this from the laboratory data. Here, we use a natural experiment to address this question. The interconnected basins of the saltern of Aigues-Mortes, France, form a metacommunity whose species composition varies in space and time. Taking advantage of this variation, we used long-term prevalence data to place the two parasites into the epidemiological categories described above (spillover, facultative multi-host, or obligate multi-host parasites). We analyzed the effects of host community composition on the presence of the parasites, isolating this effect by controlling for key environmental drivers. This resulted in both seasonal and host composition effects, which we disentangled using temperature experiments. We found that the first microsporidian is a spillover parasite, whose spillover dynamics shape its seasonal prevalence. In contrast, we were unable to identify conclusively whether the second microsporidian is a spillover or a facultative multi-host parasite, and we discuss the relative merits of our approach compared with projections based on epidemiological models.

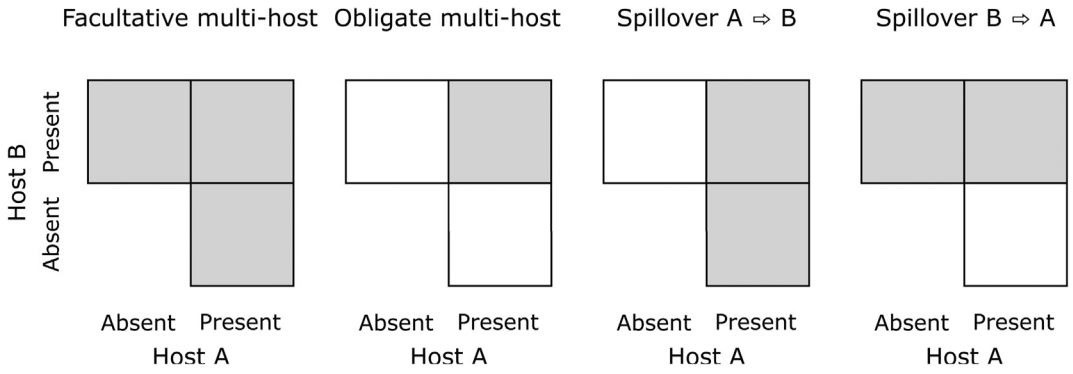

Fig. 1. Using variation in the composition of host communities to categorize multi-host parasites. Host presence can vary in space or time, creating a set of possible host communities (represented by the sets of squares). Given similar environmental conditions, the repeated occurrence (gray squares) or absence (white squares) of parasites in these communities can lead to direct conclusions about the parasite's dependence on its different hosts. Categories following Fenton et al. (2015).

2. Materials and methods

2.1. Host-parasite system

Artemia (Branchiopoda: Anostraca), also called brine shrimp, is a genus of small crustaceans whose members populate salt lakes and salterns around the world. In southern France, two *Artemia* species coexist: *A. parthenogenetica* and *A. franciscana*. *Artemia parthenogenetica* is a parthenogenetic clade native to the area, while *A. franciscana* is a bisexual species native to the New World (Thiéry and Robert, 1992; Amat et al., 2005). *Artemia franciscana* was first introduced to this region in the 1970s (Rode et al., 2013c).

Anostracospora rigaudi and *E. artemiae* are microsporidian parasites of *Artemia* (Rode et al., 2013a). Although highly prevalent in southern France (Rode et al., 2013c), they have only recently been described and little is known about their ecology. *Anostracospora rigaudi* is endemic to southern France, and therefore interacted with *A. parthenogenetica* until the introduction of *A. franciscana* (Rode et al., 2013c). In contrast, it is unknown whether *E. artemiae* is invasive or endemic in France – i.e. whether its evolutionary history is with *A. franciscana* or *A. parthenogenetica* (Rode et al., 2013c). Both species belong to a clade of microsporidians that mostly infect the intestinal epithelium of insects and crustaceans (Rode et al., 2013a). Although they belong to different genera, *A. rigaudi* and *E. artemiae* have similar life cycles. Infection occurs when ingested spores invade the gut epithelium. New spores are produced in the invaded tissue and released into the environment via the host's faeces (horizontal transmission, Rode et al., 2013a,b). In a recent laboratory study, we showed that while the parasites can complete their life cycle in both hosts, *A. rigaudi* performs well on *A. parthenogenetica* and poorly on *A. franciscana*; the reverse is true for *E. artemiae* (Lievens et al., 2018). This partial specialization is driven by high infectivity and transmission rates in the preferred host, and is associated with maladaptive virulence. In the field, *A. rigaudi* infections decrease the brooding probability of female hosts (Rode et al., 2013c); in the laboratory, both parasites affect the survival rate and reproduction of their hosts, with particularly strong effects on *A. franciscana* (Lievens et al., 2018). Previously, in a 'snapshot' sampling effort of the Mediterranean coast, *A. rigaudi* was found to be more prevalent in *A. parthenogenetica*, while *E. artemiae* was more prevalent in *A. franciscana* (Rode et al., 2013c).

We studied *A. rigaudi* and *E. artemiae* infecting *A. franciscana* and *A. parthenogenetica* in the saltern of Aigues-Mortes, in southern France. Aigues-Mortes is an active salt production site. Seawater is pumped into the saltern and into a network of shallow basins, where salinity gradually increases through solar evaporation, and finally into crystallizer ponds where salt is harvested (Supplementary Fig. S1). The total area of this network exceeds 7500 ha, with individual basins ranging from less than 1 ha to over 100 ha in size; they are generally 50–150 cm deep at our sampling sites. The major environmental factors in the saltern are temperature and salinity, both of which vary seasonally (Fig. 2A, B). *Artemia* are present year-round in large quantities, but their average density varies by more than an order of magnitude between late winter and early summer (estimated at, respectively, $\leq$1 and 10–15 individuals/L; J. P. Rullmann and P. Grillas, personal communication). The species composition of the *Artemia* community also varies seasonally. *Artemia parthenogenetica* are entirely absent in winter, but form the majority of the population in summer (Fig. 2C) (Lievens et al., 2016).

The Aigues-Mortes saltern forms a metacommunity of *Artemia* hosts with patchy species composition. The canals between basins are opened or closed as a function of the salt production process, so that there is a sporadic flow of water – and *Artemia* – between the basins (Supplementary Fig. S1). The flow of water is frequent enough to prevent genetic spatial structuring in the *Artemia* populations (Nougué et al., 2015), but irregular enough to separate *Artemia* populations on an epidemiological timescale (which is in the order of weeks, Lievens et al., 2018). Environmental factors such as salinity and food quality also vary on this timescale, leading to variation in the outcomes of inter-host competition; *A. franciscana* or *A. parthenogenetica* can outcompete one another or coexist (Browne, 1980; Browne and Halanych, 1989; Barata et al., 1996b). At any given time, therefore, adjoining basins can contain different host communities. Within basins, *Artemia* populations are well mixed (Lenz and Browne, 1991), so that we can assume that the microsporidians' spore pools are shared among the host species (cf. Fels, 2006). Each of the basins is large enough to sustain a very large *Artemia* population (>10^4), so we do not expect stochastic effects associated with small host populations to affect the microsporidians' epidemiology.

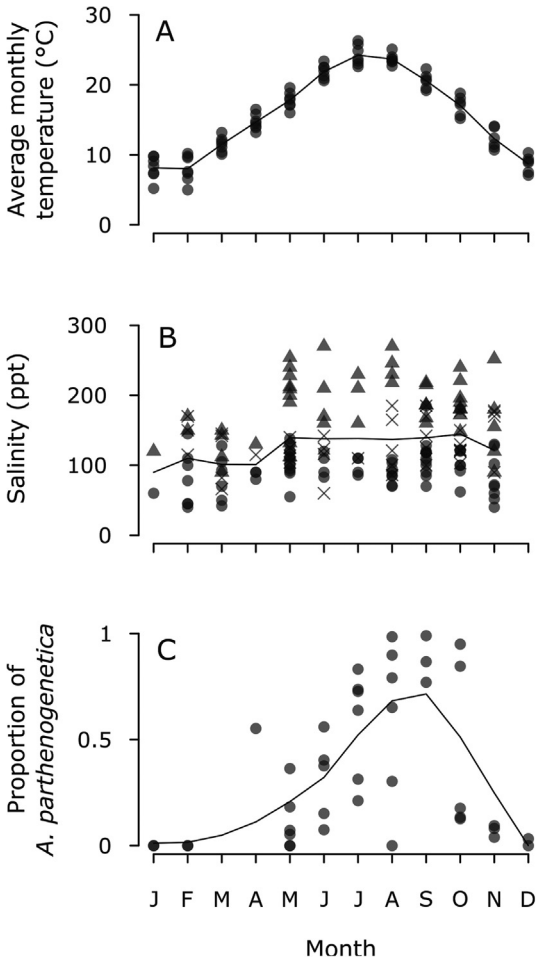

Fig. 2. Seasonality in the Aigues-Mortes saltern, France. Each point represents one data point; overlapping points shade to black. (A) Average monthly temperature between 2008 and 2015, recorded at the nearby meteorological station Le Grau-du-Roi – Repausset-Levant (Association Infoclimat, 2001, www.infoclimat.fr). The line traces the mean temperature per month. (B) Salinity, as recorded at our sampling sites between 2008 and 2015 (n = 161 observations). Triangles, crosses, and circles mark, respectively, high, medium, and low salinity sites (see Table 1); the line traces the mean salinity per month. (C) Species composition of the *Artemia* community, expressed as the proportion of the community that is *Artemia parthenogenetica* (figure adapted from Lievens et al., 2016). The line represents a second degree polynomial local regression (LOESS) fit.

2.2. Long-term field data

2.2.1. Data collection

Prevalence data was compiled from several years of *Artemia* monitoring in the Aigues-Mortes saltern. *Artemia* were collected

between 2008 and 2015 at 13 different sites (Table 1). We used shrimping nets to collect *Artemia*, netting several hundred to several thousand individuals at once. Host densities were not taken into account or recorded during collection. To avoid biasing prevalence estimates, we netted *Artemia* at random and at varied depths in the water column. We also excluded sites that had visible swarming behavior at the time of collection (swarming skews microsporidian prevalence, Rode et al., 2013b). Finally, to prevent infection-specific mortality from skewing the results, collected *Artemia* were either processed on the same day or stored in 96% ethanol and processed later.

Hereafter, we define a 'sample' as a set of *A. franciscana* or *A. parthenogenetica* collected at a given site on a given day. If *A. franciscana* and *A. parthenogenetica* co-occurred at that site on that day, we consider these to be two separate (although statistically dependent, see Section 2.2.2) samples. We identified the species of *Artemia* based on morphological differences under a binocular microscope (Perez et al., 1994). To process samples, we selected a random subset of adults to test for the presence of *A. rigaudi* and *E. artemiae*. Testing was done by PCR using species-specific microsporidian primers, following Rode et al. (2013a). We obtained microsporidian prevalence data from 117 samples: 62 samples of *A. franciscana* and 55 samples of *A. parthenogenetica* (mean n per sample = 19.0, S.D. = 14.5; Supplementary Table S1).

In addition to prevalence data, we had environmental data for each of the samples. First, we could use the sampling month as a proxy for the prevailing temperature conditions. All our sites are similarly shallow, so they have the same water temperature at any given time. Since temperatures in the saltern are very consistent across years (Fig. 2A), sampling month was a satisfactory proxy for medium-term differences in temperature. Second, we knew whether each sample came from a low, medium or high salinity site. The absolute salinity at any given site can change suddenly if the water flow in the saltern is redirected, so this measure is not well suited to the epidemiological timescales that interested us. However, the structure of the saltern means that the salinity at some sites is always lower, higher, or approximately equal to the average salinity of the saltern at that time. We classified these as low, high, and medium salinity sites, respectively (n = 7, 3, and 3 sites; Table 1, Fig. 2B). This relative classification acts as a residual of salinity after seasonal effects are taken into account, and is not sensitive to the large variability of the absolute salinity measures.

Finally, we had information on the species composition of each *Artemia* collection, i.e. which of the *Artemia* spp. was present at a given site on a given date. This information was obtained separately from the prevalence estimates, and using many more individuals. We drew a random subset of adult *Artemia* from each collection (mean sample size = 110.1, S.D. = 71.0; Supplementary Table S1) and identified these to the species level under a binocular microscope (Perez et al., 1994). Out of the 82 independent collections, most contained both *A. franciscana* and *A. parthenogenetica*, some contained only *A. franciscana*, and a few contained only *A. parthenogenetica* (n = 54, 25, and 3 collections, respectively; Supplementary Table S1).

2.2.2. Statistical analyses

The goal of our statistical analyses was to identify whether host community composition affected the prevalence of *A. rigaudi* and *E. artemiae*, while controlling for the two major environmental factors in the saltern (temperature and salinity). To do this, we analyzed the prevalence data of each microsporidian species in two steps. First, we modeled prevalence as a general function of the environment; second, we investigated the additional effect of host community composition. All analyses were performed using generalized additive mixed models (GAMMs)(in R version 3.1.3, R Core Team, 2014. R: A language and environment for statistical computing.; package "gamm4", Wood, S., Scheipl, F., 2017. gamm4: Generalized Additive Mixed Models using "mgcv" and "lme4". R package version 0.2–5.), with the number of infected versus non-infected hosts as the response variable (binomial response with logit link). Analyses with binomial GAMMs account for differences in sample size by weighting each sample proportionally to its size, and do not impose a linear relationship between the continuous covariate and the response. Model comparison was done using the corrected Akaike information criterion (AICc) (Hurvich and Tsai, 1989). Models that explain the data best have lower AICc values; ΔAICc (the difference between the AICc of the first model that does not include a given factor and the AICc of the model that best explains the data) >2 is usually considered to be good support (Burnham and Anderson, 2004). Negative ΔAICc values indicate that the factor first appears in a model with poor explanatory power.

First, we constructed models to describe each microsporidian's prevalence in the two hosts, while controlling for environmental variation. The full model was $Prevalence_{ij} = Host\ species_i + Relative\ salinity_j + s(Month_j) + Host\ species_i \times Relative\ salinity_j + s(Month_j) \times (Host\ species_i + Relative\ salinity_j) + Site_j/SiteDate_j$, where $Prevalence_{ij}$ represents the number of infected versus non-infected hosts in host species i in collection j, $s(Month_j)$ absorbs seasonal variation in temperature by applying a smoothing function to the continuous variable *Month* (the degree of smoothness is adjusted automatically), and $Relative\ salinity_j$ represents the additional variation in salinity across sites. $Site_j/SiteDate_j$ are nested random effects: $Site_j$ controls for any consistent physicochemical variation between sites (e.g. depth or size), while $SiteDate_j$ controls for the non-independence of prevalences in *A. franciscana* and *A. parthenogenetica* from the same collection (i.e. collected at the same site on the same day). We also ran a parallel set of models without the random effect $Site_j$: because working salterns regularly re-distribute the

Table 1
Sampling sites in the Aigues-Mortes saltern, France. $n_{A.f.}$ and $n_{A.p.}$ are the number of *Artemia franciscana* and *Artemia parthenogenetica* samples collected at each site.

Site	GPS coordinates	Salinity classification	$n_{A.f.}$	$n_{A.p.}$
Caitive Nord	43° 31′ 10.84″ N, 4° 14′ 26.13″ E	medium	2	3
Caitive Sud	43° 31′ 10.47″ N, 4° 14′ 26.12″ E	low	0	1
Fangouse	43° 30′ 16.04″ N, 4° 13′ 28.75″ E	high	6	0
Pont de Gazette	43° 31′ 04.63″ N, 4° 10′ 48.56″ E	low	14	11
Puits Romains	43° 30′ 17.83″ N, 4° 13′ 27.16″ E	medium	6	5
Site 1	43° 29′ 53.03″ N, 4° 14′ 23.06″ E	low	1	6
Site 3	43° 31′ 02.65″ N, 4° 14′ 29.53″ E	low	2	5
Site 4	43° 32′ 24.55″ N, 4° 13′ 25.90″ E	medium	8	9
Site 8	43° 31′ 37.17″ N, 4° 10′ 37.77″ E	low	1	3
Site 9	43° 32′ 40.31″ N, 4° 09′ 16.59″ E	high	16	8
Site 10	43° 32′ 40.10″ N, 4° 09′ 17.16″ E	high	2	2
Site 12	43° 31′ 55.66″ N, 4° 10′ 23.92″ E	low	0	1
St. Louis	43° 32′ 56.78″ N, 4° 10′ 07.95″ E	low	4	1

water between basins, it was unclear whether sites should be considered as consistent units or not. The random effects were retained in all of the compared models. The resulting optimal models are hereafter referred to as the 'general models'.

Second, we investigated whether prevalence was affected by host species composition, independently of the environmental variation already explained by our general models. We restricted our dataset to observations of *A. franciscana* because we had no power to test the effect of species composition on prevalence in *A. parthenogenetica* (the latter was only present by itself in three samples). For each microsporidian species, we used the general model from the previous section as a null model, and compared it with alternative models with added details. We modelled $Prevalence_j$ = (fixed terms of the general model) + *Presence of A. parthenogenetica*$_j$ + (fixed terms of the general model) × *Presence of A. parthenogenetica*$_j$ + $Site_j$/$SiteDate_j$, where *Presence of A. parthenogenetica* in collection *j* is our variable of interest, $Site_j$ is a random effect controlling for physical variation between sites (removed in the parallel analyses, see above), and $SiteDate_j$ is an observation-level random effect accounting for heterogeneity across samples (overdispersion, Harrison, 2015). Because *Host species*$_i$ was always *A. franciscana*, it was no longer necessary to include this fixed effect, as we did in the general models. The other fixed terms of the general model and the random effects were retained in all of the compared models.

In addition, we tested whether coinfection rates (with both *A. rigaudi* and *E. artemiae*) were significantly higher or lower than expected, as may be the case when parasites inhibit or facilitate each other, or when certain classes of hosts are more or less vulnerable. We used Cochran-Mantel-Haenszel tests (package "stats" in R version 3.1.3, R Core Team, 2014) to test the independence of *A. rigaudi* and *E. artemiae* prevalence across samples; these tests were executed separately for *A. franciscana* and *A. parthenogenetica*.

2.3. Experimental tests of microsporidian infectivity

The results of our long-term field data revealed seasonal prevalence cycles in *A. rigaudi*, correlated with the seasonal presence of *A. parthenogenetica* (see Section 3.1). To investigate whether the wintertime absence of *A. parthenogenetica* was the only driver of seasonality in *A. rigaudi*– i.e. would *A. rigaudi* be able to persist in winter if *A. parthenogenetica* was present? –, we performed two experiments testing the effects of temperature on *A. rigaudi* and *E. artemiae*. In both cases, we detected infection using the PCR protocol described by Rode et al. (2013a), and we relied on their finding that an infection is detectable 5–6 days after the host has been exposed to parasite spores.

2.3.1. Experiment 1: effect of temperature on transmission

We tested the effects of temperature on parasite transmission by exposing non-infected *A. franciscana* or *A. parthenogenetica* to *A. rigaudi* and *E. artemiae* at 15 or 25 °C. These are typical high temperatures in the saltern in March and November (15 °C) and June and September (25 °C) (Association Infoclimat, 2001, www.infoclimat.fr). The non-infected, 'recipient' hosts were adult *Artemia* spp. pulled from laboratory stock collections of Aigues-Mortes lineages. For *A. parthenogenetica*, prior evidence suggests that microsporidian infectivity depends on host genotype (Rode et al., 2013b), so the recipient hosts were collected from four clones, P6 to P9 (P6 and P7 correspond to PAM6 and PAM7 in Nougué et al., 2015). For each clone, we used females from two lines, which had been maintained separately for several generations to standardize maternal effects. We named these recipient groups P6.1, P6.2, P7.1, P7.2, P8.1, P8.2, P9.1, and P9.2. For *A. franciscana*, we formed four replicate recipient groups, F1 to F4.

From each recipient group, 10 individuals were exposed to infection at 15 °C, and 10 individuals were exposed to infection at 25 °C. Due to limited numbers of available *Artemia*, we controlled for accidental infections in the stocks (i.e. negative controls) using only five individuals from each recipient group at 15 °C and at 25 °C. The recipient individuals were infected via exposure to infected 'donor' hosts. Donor hosts were a mixed group of *A. parthenogenetica* and *A. franciscana*, collected from sites in the Aigues-Mortes saltern with high prevalences of *A. rigaudi* and *E. artemiae* (as ascertained by preliminary PCRs). Using a field-sampled group of mixed donors mimicked natural transmission conditions. The recipient groups (10 individuals) were placed in 300 mL jars filled with artificial seawater at a salt concentration of 50 g/L (Instant Ocean, Aquarium Systems, Ohio, USA). Groups of 15 donors were placed in strainers above the jars. This allowed spores to pass through, but kept donors and recipients from mixing (Rode et al., 2013b). The strainers were rotated between the jars every 45 min, so that the recipients were exposed to all donor groups (thereby controlling for any variation in prevalence or intensity of infection in the donors). Exposure lasted for 9 h, followed by a 6-day incubation period. On day 6 of the incubation period, the surviving individuals (94%) were sacrificed and tested for the presence of *A. rigaudi* and *E. artemiae* as described previously. Individuals were fed daily with *Dunaliella tertiolecta* algae ad libitum throughout the experiment.

2.3.2. Experiment 2: effect of temperature and incubation time on A. rigaudi detection

In experiment 1, both microsporidians had low transmission success at 15 °C (see Section 3.2.1). However, the factors underlying this temperature effect were unclear. The reduced transmission could have been caused by a direct effect of temperature on the microsporidians (e.g. on spore germination, Undeen et al., 1993), or by indirect effects of temperature on the ectothermic hosts. Cool temperatures lower the metabolic rate of *Artemia* (Engel and Angelovic, 1968), thereby also lowering their defecation and ingestion rates (which would have reduced the effective inoculum size, Burns, 1969; Larsen et al., 2008) and dampening their cellular metabolism (which could have slowed the accumulation of microsporidian DNA in the host, Dunn et al., 2006). Since *E. artemiae* was present in the field in winter (see Section 3.1), we could infer that its low transmission success at 15 °C must be due, at least in part, to indirect effects on the hosts. However, for *A. rigaudi*, which was not found in the field in winter (see Section 3.1), it was important to disentangle these confounding effects. To do this, we designed an experiment to compare the effects of temperature and incubation time on (detected) infectivity, while maintaining a standardized spore dose. We limited this experiment to testing *A. rigaudi* infecting *A. franciscana*.

In this experiment, we allowed *A. rigaudi* infections to incubate at 15 °C or 25 °C for different lengths of time (6 days versus 12 days; a diagram of the experiment is provided in Supplementary Fig. S2). We exposed adult *A. franciscana* from an uninfected laboratory stock population to feces containing *A. rigaudi* spores. The feces were collected from a laboratory stock of *Artemia* spp. infected with *A. rigaudi*; the spore concentration was unknown. Exposure occurred in groups: six groups of 20 hosts were placed in 50 mL of a parasite-free saline medium produced by diluting concentrated, autoclaved brine (Camargue Pêche, France) to 90 ppt with deionized water. Fecal solution (2.8 mL) was added to each group. Four groups were exposed at 15 °C, while two groups were exposed at 25 °C. Exposure lasted 2 days, during which time all spores could be ingested (Reeve, 1963). After 2 days, hosts were separated and each individual was placed in a hemolymph tube containing 2.5 mL of brine; this prevented between-recipient infections later in the experiment. The infection was allowed to

incubate at the exposure temperature for 4 days, after which half of the surviving hosts from each group were sacrificed. At the same time point, two of the groups exposed at 15 °C were moved to 25 °C. This let us separate the effects of temperature on infection success from the effects of temperature on the accumulation of parasites within the host. After a further 6 days of incubation, the surviving hosts (93%) were sacrificed and tested for the presence of *A. rigaudi*. Throughout the experiment, hosts were fed 0.25 mL of *Tetraselmis chuii* solution ($6.8*10^9$ cells/L, Fitoplancton marino, Spain) every 2 days.

2.3.3. Statistical analyses

Statistical analyses of the experiments were performed using generalized linear mixed models (package "lme4", Bates et al., 2015; in R version 3.1.3), with the number of infected versus non-infected hosts as the response variable (binomial response with logit link). The significance of the predictors was tested using likelihood ratio tests; *P* values below 0.05 were considered significant.

For experiment 1, we analyzed the probability of detecting an infection separately for *A. rigaudi* and *E. artemiae*. Fixed effects included *Temperature*, a *Species/Genotype* factor, and their interaction. As we expected to find differences among the *A. parthenogenetica* clones (Rode et al., 2013b), but could not distinguish between the mixed-together *A. franciscana* families, the *Species/Genotype* factor comprised five levels: *A. franciscana*, *A. parthenogenetica* P6, *A. parthenogenetica* P7, *A. parthenogenetica* P8, and *A. parthenogenetica* P9. We included *Recipient group* as a random effect to control for shared genetic and environmental effects.

For experiment 2, our statistical analyses used *Exposure temperature*, *Periods incubating at 15 °C*, and *Periods incubating at 25 °C* as fixed effects (with one period equal to 6 days), with *Host group* as a random variable controlling for pseudo-replication. Since the sensitivity of our PCR was fixed, an increase in detectability over time reflects an increase in the quantity of parasite DNA present in the host (i.e. a higher parasite intensity).

2.4. Data accessibility

Data and analyses are published in the Dryad repository, doi: 10.5061/dryad.sg4m5h0.

3. Results

3.1. Long-term field data

First, we described the prevalence dynamics for *A. rigaudi* and *E. artemiae* as a function of the environment (Fig. 3, Supplementary Table S2). *Anostracospora rigaudi* was strongly seasonal: it was highly prevalent from August to October, but absent in winter (*Month* effect, $\Delta AICc \geq 44.8$; Fig. 3A, B). These seasonal dynamics were not different in the two hosts, but its prevalence was higher in *A. parthenogenetica* (effect of *Host species*, $\Delta AICc \geq 119.2$; compare Fig. 3A and B). The prevalence of *A. rigaudi* was also higher in low and medium salinity sites (*Relative salinity* effect, $\Delta AICc \geq 6.8$; Supplementary Fig. S3). The prevalence of *E. artemiae* was highly variable, but consistently higher in *A. franciscana* (effect of *Host species*, $\Delta AICc \geq 114.4$; compare Fig. 3D and C). Neither of the environmental drivers were well supported for this parasite (*Month* effect, $\Delta AICc \leq -3.5$; *Relative salinity* effect, $\Delta AICc \leq -4.3$). Running the same models without the random effect *Site* generated equivalent results (Supplementary Table S3).

Next, we added details about the host community composition to the general models, investigating the effect of *A. parthenogenetica* on the prevalence of the microsporidians in *A. franciscana* (Fig. 4, Supplementary Tables S4, S5). For *A. rigaudi*, the species composition had a clear effect ($\Delta AICc \geq 2.3$; $\Delta AICc \geq 5.0$ in models without a random effect of *Site*): in the absence of *A. parthenogenetica*, the prevalence of *A. rigaudi* was almost always 0%, and never higher than 10% (Fig. 4B). In contrast, in the presence of *A. parthenogenetica* the prevalence could be very high, and the seasonal dynamics found in the general model reappeared (Fig. 4A). Importantly, this effect occurred both among and within sites (Supplementary Table S1). For *E. artemiae*, there was little support for an effect of species composition ($\Delta AICc \leq -1.2$; $\Delta AICc \leq -1.4$ in models without a random effect of *Site*).

Finally, coinfection rates varied from 0% to 83% in our samples (Supplementary Table S1). Infection with *A. rigaudi* and *E. artemiae* was statistically independent for *A. parthenogenetica* (Mantel-Haenszel $\chi^2(1) < 0.1$, $P = 0.84$, common odds ratio = 0.8), but was positively associated for *A. franciscana* (more coinfection observed than expected; Mantel-Haenszel $\chi^2(1) = 9.6$, $P = 0.002$, common odds ratio = 2.7).

3.2. Experimental tests of microsporidian infectivity

3.2.1. Experiment 1: effect of temperature on transmission

The P8 genotype of *A. parthenogenetica* was previously infected (all controls and exposed hosts tested positive for *A. rigaudi*). The same was true of the F4 replicate of *A. franciscana* (all controls and half of exposed hosts tested positive for *E. artemiae*). These two recipient groups were removed from the analysis. Surprisingly, the previous infections appeared to have an inhibitory effect: none of the exposed P8s were infected with *E. artemiae* at the end of the experiment ($n = 37$), and none of the exposed F4s were infected with *A. rigaudi* ($n = 15$).

Temperature had a clear effect on the probability of infection of both *A. rigaudi* and *E. artemiae*; neither caused many infections when exposure occurred at low temperatures ($\chi^2(1) = 136.3$ and 46.5, $P < 0.001$ in both cases; Fig. 5). *Anostracospora rigaudi* infected all three genotypes of *A. parthenogenetica* and *A. franciscana* equally well ($\chi^2(3) = 0.8$, $P = 0.85$), but infectivity was dependent on *Species/Genotype* in *E. artemiae* ($\chi^2(3) = 18.1$, $P < 0.001$; Fig. 5B). Post-hoc Tukey tests indicated that *A. franciscana* and P9 were equally susceptible to *E. artemiae*, while P6 and P7 were much less susceptible ($z \leq -2.9$, $P < 0.01$). There were no significant interaction effects between temperature and *Species/Genotype*.

3.2.2. Experiment 2: effect of temperature and incubation time on A. rigaudi detection

The probability of detecting *A. rigaudi* in *A. franciscana* – i.e. the infection intensity – increased significantly with incubation period at 25 °C, but not at 15 °C ($\chi^2(1) = 31.9$ and 1.9, $P < 0.001$ and $P = 0.18$, respectively; Fig. 6). There was no significant effect of the exposure temperature on infectivity ($\chi^2(1) = 0.0$, $P = 0.91$). Therefore, the apparent reduction in infectivity of *A. rigaudi* at 15 °C during experiment 1 was at least partly caused by slower parasite reproduction inside the hosts, delaying its detectability. The lower rates of detection after 6 days of incubation at 25 °C in this experiment compared with experiment 1 could be explained by a lower initial spore dose.

4. Discussion

When a parasite infects multiple host species, we cannot gain an adequate understanding of its epidemiology and evolution without knowing which of the host species actually contributes to its transmission and maintenance. In this study, we exploit natural variation in the composition of *Artemia* host communities to identify their microsporidian parasites as either spillover, faculta-

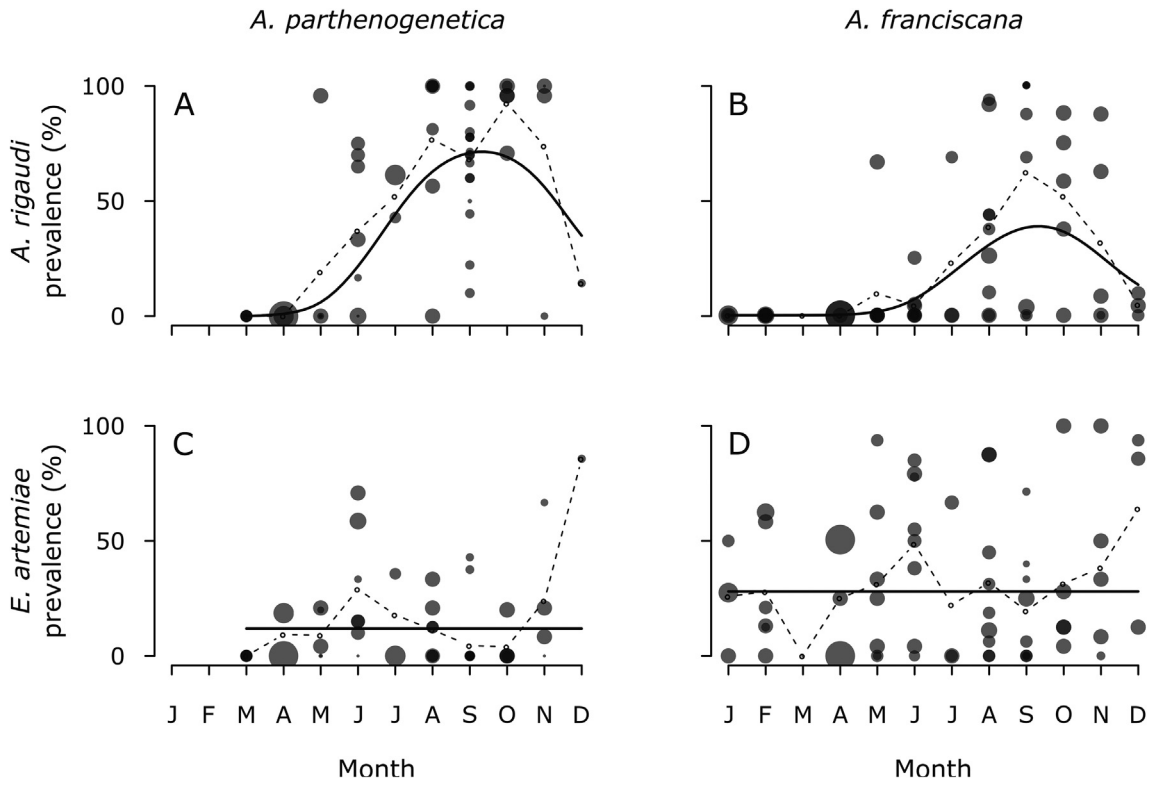

Fig. 3. Seasonal prevalence of the parasites *Anostracospora rigaudi* (A, B) and *Enterocytosopra artemiae* (C, D) in their hosts *Artemia parthenogenetica* (A, C) and *Artemia franciscana* (B, D). Solid dots are observed data; the area of the dot represents the number of individuals in the sample. Overlapping dots shade to black. Solid line: predictions of the general model, represented by the marginal mean (obtained here by averaging the predictions for all random effects). Using the marginal mean was necessary to compensate for the high variability between samples. Dashed line with open circles: mean prevalence across samples. There is no prevalence data for *A. parthenogenetica* in January and February because this host is absent in winter.

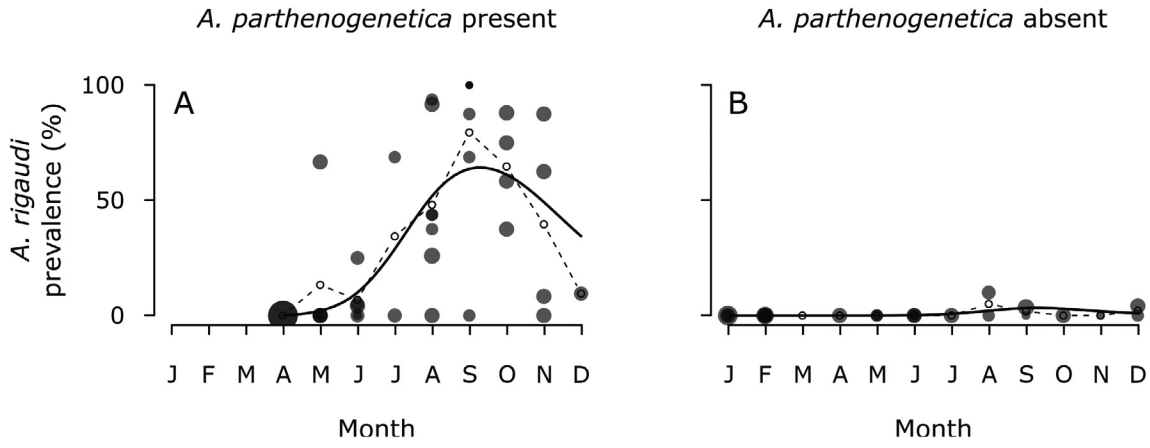

Fig. 4. Decomposition of Fig. 3B: prevalence of *Anostracospora rigaudi* in *Artemia franciscana* when *Artemia parthenogenetica* is present (A) or absent (B). Solid dots are observed data; the area of the dot represents the number of individuals in the sample. Overlapping dots shade to black. Solid line: predictions of the best detailed model, represented by the marginal mean (obtained here by averaging the predictions for all random effects). Using the marginal mean was necessary to compensate for the high variability between samples. Dashed line with open circles: mean prevalence across samples.

tive multi-host, or obligate multi-host parasites (Fenton et al., 2015). Our primary result is that *A. rigaudi* is a spillover parasite, whose high infectivity in both hosts causes pseudo-endemic dynamics. This result demonstrates that even competent hosts with a high abundance and high susceptibility may dilute a parasite population. Secondly, we show that *E. artemiae* may be a spillover or a facultative multi-host parasite, and speculate that it is the first. Finally, we consider the link between the microsporidians' host specificity and seasonal dynamics, and discuss the merits of our method.

Our long-term prevalence data revealed that *A. rigaudi* is a spillover parasite: it cannot persist on *A. franciscana* in the absence of *A. parthenogenetica* (Fig. 4). The presence of the parasite in *A. franciscana* must therefore be dependent on regular re-introductions from *A. parthenogenetica*, its maintenance host. This result is robust to variation in temperature (the effect can be found in every season, Fig. 4) and salinity (the effect is found across all salinity categories, results not shown), which are the main environmental factors that affect this system. Under the relevant natural condi-

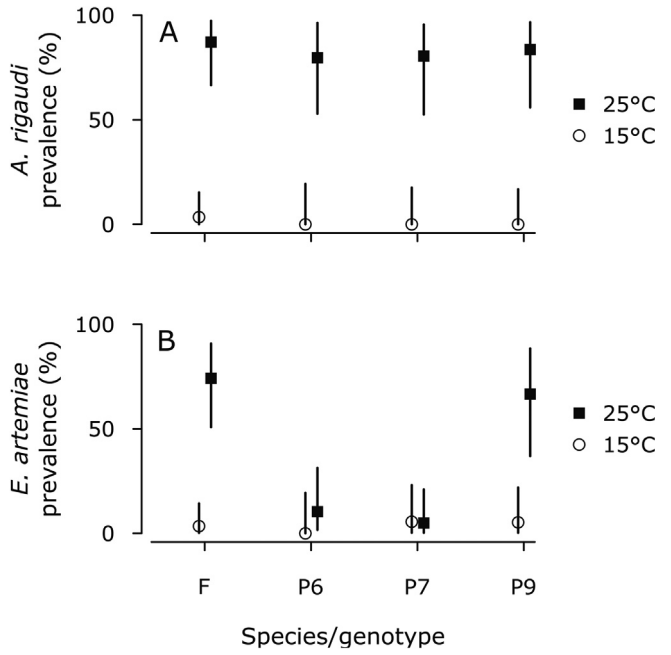

Fig. 5. Infectivity of *Anostracospora rigaudi* (A) and *Enterocytospora artemiae* (B) as a function of temperature and host type (experiment 1). Individuals were sacrificed and tested after 6 days of exposure and incubation at 15 °C or 25 °C. Species/genotypes were *Artemia franciscana* (F) and *Artemia parthenogenetica* isofemale lines P6, P7 and P9. Vertical lines represent the 95% confidence intervals; the final *n* for each value ranges from 23 to 29 for host species F and from 16 to 20 for each line of host species P.

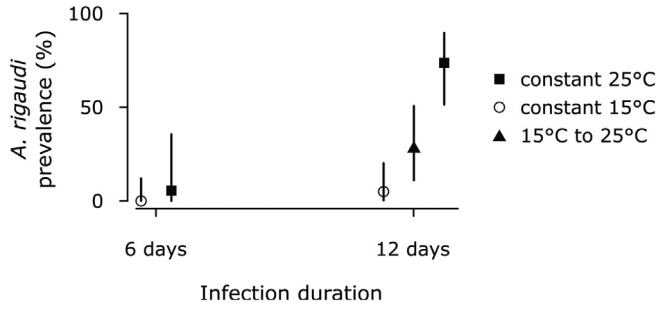

Fig. 6. Detection of *Anostracospora rigaudi* infections in *Artemia franciscana* as a function of temperature (experiment 2). Groups of individuals were exposed to *A. rigaudi* and maintained at 15 °C or 25 °C for six or 12 days, after which they were sacrificed and tested. Two groups were exposed and maintained at 15 °C for the first 6 days, and then moved to 25 °C for the remaining 6 days. Vertical lines represent the 95% confidence intervals; from left to right, *n* = 36, 18, 20, 18, 19.

tions, populations containing only *A. franciscana* are unable to maintain *A. rigaudi*.

Parasites are unable to persist in a host species if that host provides little or no transmission, which may occur if its susceptibility, abundance, and/or lifetime spore production are low (Dobson, 2004; Streicker et al., 2013). Our current and previous experiments ruled out the first possibility for *A. franciscana* and *A. rigaudi*, as both hosts were quite susceptible to the microsporidian (Fig. 5A) (Lievens et al., 2018). The second possibility, that sites containing only *A. franciscana* have consistently lower host densities, we also consider to be unlikely. Although the dataset used here does not contain demographic information, both observations and available evidence show that *A. franciscana*-only sites can reach densities similar to sites containing both hosts (J. P. Rullmann and P. Grillas, personal communication). Instead, *Artemia* biomass is mainly constrained by food availability (Browne, 1980), temperature (Barata et al., 1996a), and salinity (Wear and Haslett, 1986), which are seasonal environmental factors (see Fig. 2A, B). The third possibility is supported by experimental evidence. The lifetime spore production of *A. franciscana* infected with *A. rigaudi* is indeed very low (Lievens et al., 2018), a maladaptation caused by over-virulence (Woolhouse et al., 2001). We therefore conclude that *A. franciscana* is a spillover host for *A. rigaudi* because infected hosts produce few spores.

The contrast between the generalist infectivity of *A. rigaudi* and its status as a spillover parasite is particularly interesting. At first glance, its high infectivity in the two hosts, as measured in the laboratory and reflected in the field prevalences (Fig. 3A, B, 5A), might tempt us to conclude that *A. rigaudi* is a generalist parasite. Only a more detailed analysis of the field data belies this generalist infectivity, and reveals the hidden host specificity of *A. rigaudi*. This result highlights the dangers of using only infectivity as an indicator of parasite specialization and long-term success (Agosta et al., 2010; Lange et al., 2015). Furthermore, the high infectivity of *A. rigaudi* in *A. franciscana* means that spillovers from *A. parthenogenetica* to *A. franciscana* occur frequently enough that the parasite appears to be independently present in both hosts (Fig. 3A, B). Some authors have termed such frequent spillovers 'apparent multi-host' or 'pseudo-endemic' dynamics (Fenton and Pedersen, 2005; Viana et al., 2014; cf. Dobson, 1995; Rhodes et al., 1998), terms which explicitly indicate the difficulty of distinguishing such dynamics from true endemism. This makes the danger of misinterpreting the epidemiology and evolution of pseudo-endemic parasites very high if their community context is not investigated. *Anostracospora rigaudi* itself is a good example of this problem. Based on previously available information, an earlier paper proposed that *A. rigaudi* could overwinter by infecting the invasive *A. franciscana*, thereby increasing its negative impact on the population of native *A. parthenogenetica* (a process known as "spillback", Rode et al., 2013c). In contrast, our current conclusion suggests that *A. franciscana* individuals act as inhibitory hosts, absorbing more *A. rigaudi* spores than they produce (Holt et al., 2003), and thereby diluting the effect of *A. rigaudi* on *A. parthenogenetica* (Ostfeld and Keesing, 2000; Hall et al., 2009). Overall, *A. rigaudi* acts as a cautionary example against extrapolating parasite dynamics from single traits, by showing that even a highly abundant, highly susceptible host species can be poor at maintaining its parasites. So poor, in fact, that the opposite is achieved – a net loss of the parasite in the community.

We identified the second microsporidian, *E. artemiae*, as either a spillover or a facultative multi-host parasite, but our prevalence data was insufficiently powerful to distinguish between the two possibilities. We were unable to analyze the ability of *E. artemiae* to persist on *A. parthenogenetica* because only three of our field collections did not contain *A. franciscana*. Although those three samples were *E. artemiae*-free, this may have been due to chance. Based on our laboratory experiments, however, we suspect that *E. artemiae* is also a spillover parasite, in this case from *A. franciscana* to *A. parthenogenetica*. Lievens et al. (2018) demonstrated that the lifetime spore production of *E. artemiae* in *A. parthenogenetica* and *A. rigaudi* in *A. franciscana* are similarly low. *Enterocytospora artemiae* is also a very poor infector of *A. parthenogenetica* (Lievens et al., 2018). This is confirmed by the high temperature findings of experiment 1, although it is interesting to note that the effect only arises when averaging over host genotypes (Fig. 5B), consistent with the genotype-dependent prevalence data collected by Rode et al. (2013b) in natural populations. The spillover effect of *E. artemiae* might therefore be even stronger than that of *A. rigaudi*, as the latter is less specifically infective. In the absence of conclusive evidence, however, this microsporidian serves as an excellent example of the difficulty of using observational data to identify host contributions to parasite persistence.

Identifying the hosts that contribute to the persistence of *A. rigaudi* and *E. artemiae* also provides a lens through which to interpret their seasonality. It is clear that the seasonal prevalence patterns of *A. rigaudi* and *E. artemiae* (Fig. 3) are linked to the seasonal changes in host community composition (Fig. 2C). The maintenance host of *A. rigaudi*, *A. parthenogenetica*, is entirely seasonal, being completely absent in winter and highly prevalent in late summer and autumn; logically, therefore, *A. rigaudi* is also strongly seasonal. In contrast, *E. artemiae* is able to persist throughout the year, with no overarching seasonal prevalence pattern. By testing infection success at warm and cool temperatures, and comparing the two parasites, we can gain further insight into the absence of *A. rigaudi* in winter. Although low temperatures did slow parasite growth within the host when tested in *A. rigaudi* (Fig. 6), they did not make infection impossible for either parasite (*A. rigaudi*: filled triangle in Fig. 6; *E. artemiae*: open circles in Fig. 5B), and clearly do not preclude persistence of *E. artemiae* (Fig. 3D). Therefore, it is possible that *A. rigaudi* could persist in winter, if given the opportunity to do so by the presence of *A. parthenogenetica*. We therefore conclude that the general prevalence patterns of *A. rigaudi* and *E. artemiae* are predominantly shaped by host specialization, and not by environmental factors.

The seasonal cycles of *A. rigaudi* in its spillover host *A. franciscana* are of particular interest (Fig. 3B). These are clearly caused by the seasonal presence of the maintenance host *A. parthenogenetica*, which enforces seasonality on *A. rigaudi* and therefore on the frequency of spillovers. Seasonal cycles in prevalence occur in many host-parasite systems, and are often explained by factors such as climate, host behavior, and host immunity (Hosseini et al., 2004; Duffy et al., 2005, 2009; Grassly et al., 2005; Altizer et al., 2006; Lass and Ebert, 2006). In contrast, our study presents a rare example of seasonal infections within a focal host (*A. rigaudi* in *A. franciscana*) being caused by the seasonality of an entirely different host (*A. parthenogenetica*). We know of one other description of such spillover-driven seasonality. Amman et al. (2012) investigated the prevalence of Marburg virus in bats, which causes severe hemorrhagic disease when it spills over to humans. They found that viral infections in bats peaked during birthing seasons, and that 83% of spillovers to humans occurred during these peaks. Such cases highlight the interconnected nature of communities hosting multi-host parasites, and are obviously of particular interest for the establishment of control strategies.

In this study, we used variation in the composition of natural host communities to investigate how each host contributes to the maintenance of a shared parasite. The strength of this approach is illustrated by the results for *A. rigaudi*: we obtained direct evidence that under natural conditions, it is a spillover parasite dependent on *A. parthenogenetica* and unable to persist in *A. franciscana*. This conclusion, which we could not have drawn from laboratory experiments alone, has crucial consequences for our interpretation of the dynamics and evolution of both parasite and host. On the other hand, the results for *E. artemiae* highlight an important weakness of the method, namely that all possible combinations of communities must be sampled in order to obtain conclusive evidence. If one combination does not occur in the field, this constraint is inescapable. In this case, methods based on the construction of full epidemiological models, such as those described by Fenton et al. (2015), may be more useful. These methods first quantify each host's contribution to parasite transmission using observed data on host abundance, parasite prevalence, and parasite shedding; they then infer the consequences for persistence from these results. While these techniques require the accurate measurement of all relevant parameters and an adequate mathematical description of the epidemiology, they are not dependent on wide-scale sampling across communities. Such methods are therefore useful where large-scale sampling is difficult or community composition is invariable, while the natural experimental approach could be particularly suited to highly variable and regularly sampled multi-host systems (e.g. *Daphnia*, Ebert, 2008). Independently of the method used, these (and our) studies consistently demonstrate the importance of studying a parasite within its entire host community before making inferences about its host specificity, epidemiological drivers, and selection environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.01.002.

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