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Population-level dynamics in experimental mixed infections: evidence for competitive exclusion among bacterial parasites of *Paramecium caudatum*

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Parasites frequently share their host populations with other parasites. However, little is known about how different parasites respond to competition with diverse competitor species in the within-host and between-host environments. We explored the repeatability of competition by simultaneously exposing microcosm populations of the ciliate *Paramecium caudatum* to pairs of parasites from the *Holospora* species complex (*H. undulata*, *H. caryophila* and *H. obtusa*). We measured how competition affected the persistence and prevalence of each compared to single infections, across three host genotypes. Three weeks post-inoculation we identified the presence of each parasite using fluorescence in situ hybridisation (FISH). Competitive exclusion (62/72) was more common than co-existence (10/72) in populations inoculated with two parasites. There was a clear pattern of competitive superiority, with *H. caryophila* persisting in all doubly inoculated populations (with either *H. undulata* or *H. obtusa*), and *H. undulata* tending to exclude *H. obtusa*. This mirrored infection success in single infections, with *H. caryophila* having a higher infection prevalence in single inoculations, followed by *H. undulata* then *H. obtusa*. The probability of persistence in co-inoculations did not change across the different host genotypes, and prevalence was the same as in single infections. Our results are consistent with superinfection models, which assume the competitive exclusion of parasites upon contact within the same host. Furthermore, such non-random competitive epidemiological dynamics, where one parasite always wins, may be of interest for public health management, especially if the winning parasite is avirulent, as is seemingly the case here.

Keywords: co-infection, epidemiology, *Holospora*, host–parasite, microcosm

Introduction

Parasitism is a ubiquitous life-style, and in most, if not all, communities, of hosts are faced with multiple parasite strains and species. This sets the stage for complex interactions, namely competition between parasites for access to, or for resources within, hosts (Read and Taylor 2001, Mideo 2009, Alizon et al. 2013, Rodrigues et al. 2016).

For instance, infection with one parasite can either increase or decrease the probability of infection with a second (Telfer et al. 2010, Knowles et al. 2013, Halliday et al. 2017). Furthermore, within-host interactions can increase (Stein 2011, Lass et al. 2012, Susi et al. 2015a) or decrease (Duncan et al. 2015) the production of transmission stages. Thus, interactions between parasites can modify infection outcomes and thereby influence epidemiological dynamics and long-term evolution (Alizon et al. 2013, Rodrigues et al. 2016).

However, uncovering the link between individual host-level competition and population-level dynamics is not straightforward (Alizon et al. 2013, Hellard et al. 2015). Many laboratory studies compare parasite growth in singly and coinfected hosts, which is used as a proxy for potential transmission to new hosts (Alizon et al. 2013). Some have gone a step further and measure the transmission of parasites from coinfected individuals to new hosts. For example, de Roode et al (2005) found higher transmission of a more virulent, than avirulent, malaria strain from coinfected mice to mosquitoes; McCullers et al. (2010) reported elevated levels of *Streptococcus pneumonia* transmission from ferret hosts also infected with the influenza virus.

Alternatively, some studies of natural populations use correlative measures of parasite co-occurrence to detect the impact of parasite interactions on epidemiology (Lello et al. 2004, Telfer et al. 2010, Clark et al. 2016). Others have used perturbation experiments, whereby hosts are cured of one type of parasite and changes in infection patterns are observed through time (Knowles et al. 2013, Ezenwa and Jolles 2015). More recently, the coupling of field observations with common garden experiments has provided insights into how within-host parasite interactions impact epidemics (Johnson and Hoverman 2012, Susi et al. 2015a, Halliday et al. 2017). One showed that the release of transmission stages of a fungal parasite was higher from hosts coinfected with multiple strains and, at the same time, observed more severe parasite epidemics in populations with higher frequencies of coinfected hosts (Susi et al. 2015a). Another revealed that epidemic size for three different fungal parasites, as well as their within-host growth in a grass host, depended on the sequence of parasite arrival (Halliday et al. 2017). These examples highlight that local processes can transcend the within-host environment, with far reaching epidemiological consequences.

Nevertheless, still few experimental studies investigate how interactions between different parasite species shape epidemiology under controlled conditions over multiple host generations. Furthermore, little is known about the repeatability of responses when a given parasite is confronted with different competitor species.

We inoculated microcosm populations of the ciliate *Paramecium caudatum* with pairwise combinations of three bacterial parasites, of three different species, from the *Holospora* species complex (Boscaro et al. 2013): *H. caryophila*, *H. undulata* and *H. obtusa* (referred to from hereon in as *'Caryophila'*, *'Undulata'* and *'Obtusa')*. *Caryophila* and *Obtusa* both reside in the vegetative macronucleus of *Paramecium*, whereas *Undulata* resides in the generative micronucleus. Both field and laboratory coinfections in *Paramecium* have been reported for a variety of bacteria (Görtz and Fokin 2009), including the *Holospora* species used here (Fokin et al. 2004).

After three weeks of unconstrained, natural epidemiological dynamics we compared persistence (absence or presence in the population) and the prevalence of each of the *Holospora* parasite species in doubly and singly inoculated *Paramecium* microcosm populations, across three different host genotypes. We extrapolated expected frequencies of coexistence and coinfection from the observed infection success of each parasite in single inoculations. As such, expected frequencies are based on the assumption that parasites act independently of one another. The following questions were addressed: 1) are parasite persistence and prevalence different in single versus double inoculation treatments? Under independent action of parasites, we expected that overall levels of infection, the frequency of population-level coexistence and within-host coinfection could be predicted from infection success of each parasite in single-inoculation treatments. 2) Does persistence or prevalence change according to the identity of the competitor species? We hypothesised that coinfection might be more likely in double inoculations with *Undulata* because it infects a different organelle (micronucleus) to the other two parasite species (macronucleus). 3) How does host genetic background change parasite persistence and prevalence? Host genotype-by-parasite species interactions for infection success may lead to different outcomes of competitive interactions between parasites, such that higher levels of infection on a given genotype may be linked with higher competitivity.

Material and methods

Biological system

The host

Paramecium caudatum, a freshwater ciliate, is maintained in the laboratory in a growth medium made from dried, organic lettuce (1 g per 1.5 l of Volvic mineral water) and the food bacteria *Serratia marcescens* (Nidelet and Kaltz 2007). Paramecia are characterised by a nuclear dimorphism, with a diploid micronucleus and a polyploid macronucleus. The micronucleus is only active during sexual reproduction (germline function), while all gene expression during asexual stages occurs in the macronucleus (somatic function). Reproduction in this experiment was asexual (clonal), through binary fission of cells, with mitotic division of the micronucleus and amitotic division of the macronucleus. Clonal populations of three *Paramecium* genotypes were used: *CRA* (originating from Poland), *OB* (Germany) and *M3* (unknown origin, isolated from a commercial protozoan mix).

The parasites

We used three bacterial *Holospora* species: *H. undulata*, *H. obtusa* and *H. caryophila*, referred to as *'Undulata'*, '*Obtusa*' and '*Caryophila*' hereafter. Species of the genus *Holospora* are obligate parasitic endosymbionts of their *Paramecium* hosts.

Undulata and *Obtusa* are specific to *P. caudatum*, *Caryophila* also infect*s P. biaurelia* (Görtz and Fokin 2009). These gram-negative bacteria have the same infection life cycle, involving two morphologially and functionally different cell types: the short 'reproductive forms' and the elongated 'infectious forms' (Görtz and Fokin 2009). *Paramecium* ingest infectious forms when feeding, which enter a food vacuole before being transferred to either micro- or macronucleus, depending on the species. Once in the nucleus, they differentiate into reproductive forms, which multiply inside the nucleus. Vertical transmission of reproductive forms occurs during cell division, and horizontal transmission by the release of infectious forms in the medium during host cell division or following host death. These three species are easy to distinguish because of the size of infectious forms and the compartment they occupy within the host. *Obtusa* has the largest infectious forms, 20 µm (Görtz and Fokin 2009) and *Caryophila* the smallest, 5 µm (Görtz and Fokin 2009). These two *Holospora* species infect the same host compartment, the macronucleus. *Undulata* infects the micronucleus and the infectious forms are 15 µm (Görtz and Fokin 2009).

The *Undulata* inoculum was prepared from infected long-term mass cultures of different *P. caudatum* genotypes, originally all infected with a single *Undulata* strain, derived from a single infected individual from a pond in Germany (provided by H. D. Görtz). The *Caryophila* inoculum originated from an isolate infecting the *P. biaurelia* strain *FGC3,* which was subsequently propagated on mass cultures of different *P. biaurelia* and *P. caudatum* genotypes. The *Obtusa* inoculum was prepared from a single infected *P. caudatum* mass culture, provided by M. Fujishima (Yamaguchi Univ., Japan).

Inocula were prepared by centrifuging infected *Paramecium* cultures to concentrate cells in a pellet. These cells were then vortexed in 1.5 ml Eppendorf tubes containing 1 mm glass beads to break cell membranes and release infectious forms. The concentration of infectious forms of *Obtusa* and *Undulata* were determined in a haemocytometer (200× magnification) and adjusted to 310 infectious forms μl^{-1} by dilution with sterile water. Because of their small size, precise quantification of *Caryophila* infectious forms was difficult; approximate inoculation concentration was estimated to be 500–1000 μl^{-1}. Per-propagule infection probability may differ between parasite species (Duncan et al. 2015), making it difficult a priori to standardise comparisons of infectivity in an inoculation experiment, even if doses are adjusted. In our study, additional tests showed that the relative difference in infection success between *Undulata* and *Obtusa* remained approximately constant, when the infectious dose was halved (Supplementary material Appendix 2 Fig. A1), suggesting a dose-independent difference in infectivity between the two species. Along the same lines, other experiments show that *Caryophila* is highly infectious even at low doses (Supplementary material Appendix 2 Fig. A1); this indicates that *Caryophila*'s superior infectivity observed in the present experiment is not exclusively due to the higher dose administered.

Infection experiment

We compared infection success of each parasite in single inoculations and in pairwise double inoculations in microcosm populations of each of the three host genotypes. Thus, our experiment comprised six inoculation treatments: single inoculation of each parasite (*Caryophila*, *Undulata*, *Obtusa)* and all three possible double inoculations (*Caryophila* and *Undulata*, *Caryophila* and *Obtusa, Undulata* and O*btusa*). Each inoculation treatment was performed on each host clone, with nine microcosm replicates for the CRA and OB clones, respectively, and six replicates for M3 clone (143 replicates in total; Fig. 1).

A replicate microcosm consisted of 350 µl of a given *Paramecium* clone (ca 120 cells), placed in a 1.5 ml Eppendorf tube. Inoculations were performed by adding 125 µl of each parasite in the single and double inoculation treatments. In the single infection treatment, 125 µl of water was also added to equalise volumes across treatments. On day 7 post infection, all populations were fed 500 µl of the lettuce medium. On day 14, all populations were transferred to 15 ml Falcon

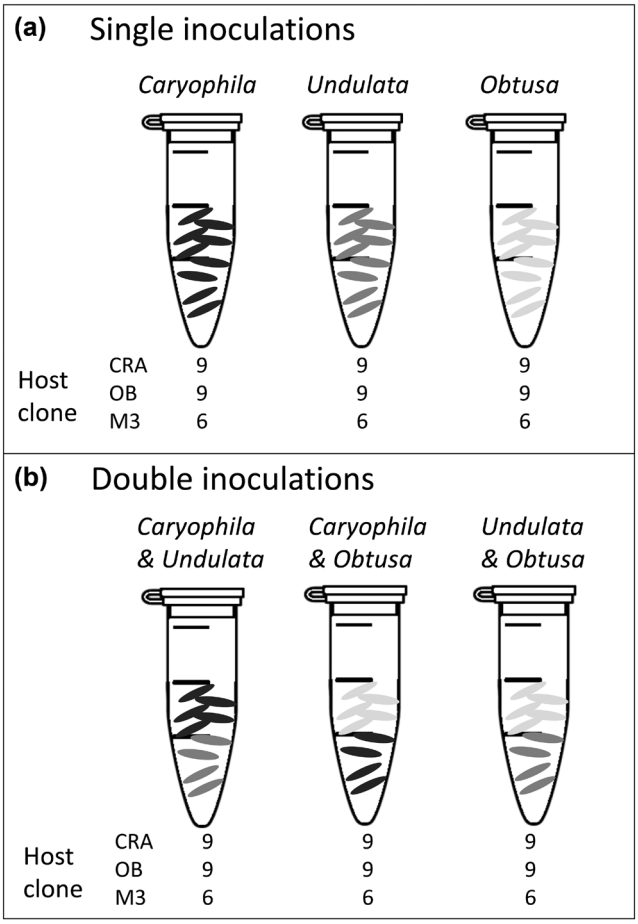

Figure 1. Experimental design for single and double inoculation treatments of *Holospora caryophila*, *H. undulata* and *H. obtusa*, tested on three *Paramecium caudatum* clones. Numbers indicate the number of replicate microcosms.

tubes and fed again with 1 ml of medium. Three weeks post inoculation, we determined the infection status and prevalence of each parasite in each replicate population using fluorescence in situ hybridisation (FISH; Supplementary material Appendix 1). Approximately 50 *Paramecium* cells (note that transfer of 50 cells results in the fixation of ~20 cells) were removed from each population and washed in Dryl's solution. *Paramecium* were then transferred to a SuperFrost Ultra microscope slide in 20 µl of Dryls solution and fixed with 20 µl of 4% paraformaldehyde solution for 6 min. Slides were then serially washed in 50%, 80% and 99,8% ethanol baths, each slide being placed in each bath for 10 min before being air dried. FISH was performed following the protocol of Manz et al. (1992) using a probe detecting most bacteria (EUB338I; Altmann et al. 1990). Twenty µl of hybridisation buffer (45 ml NaCl (5 M), 5 ml Tris-HCl (1 M pH 8), 250 µl SDS (10%), 199.75 ml H_2O) was placed on the slides with 2 µl of probe (50 ng μl^{-1}) with a cover slip. Hybridisation was completed by placing slides in the oven at 46°C for 3–18 h. The cover slip was then removed and slides were washed for 20 min in washing buffer (90 ml NaCl (5 M), 10 ml Tris-HCl (1 M pH 8), 500 µl SDS (10%), 399.5 ml H_2O), rinsed in water and mounted in 50% glycerol. In the double inoculation treatments, *Undulata* can be distinguished from the other two parasites because it infects the micronucleus, and *Obtusa* from *Caryophila* by size (Supplementary material Appendix 2). Across all microcosm replicates, the mean number of individuals inspected for infection was 19.8 (± 0.4 SE, median: 20; 80% range 12–26).

Statistical analysis

We used generalised linear models with a binomial error structure and logit link function to investigate variation in parasite infection prevalence (proportion of infected individuals in singly or doubly inoculated populations) and parasite coexistence (presence of both parasites versus only one parasite present in a doubly inoculated population). Explanatory factors included parasite species identity (*Caryophila, Undulata, Obtusa*), parasite combination in double inoculations (*Caryophila* and *Undulata*, *Caryophila* and *Obtusa*, *Undulata* and *Obtusa*) and host clone identity (*CRA, M3, OB*). Note that, for the double inoculation treatments, we used the total infection prevalence, which is based on the sum of all infected individuals in a population.

For the double inoculation treatments, our main analyses tested whether observed levels of coexistence and infection prevalence differed from expected. The calculation of these expected values was based on infection success in single inoculations and assumed independent action of parasites when coinoculated. The details of these calculations are given in the Supplementary materials (Supplementary material Appendix 2). Briefly, the rationale of a simple bootstrap procedure (n = 100 runs) was to create a distribution of expected outcomes of coexistence, taking into account the finite sample sizes of the FISH technique and thus possible non-detection of a parasite, even if present in the population. We used a Fisher's exact test for an overall comparison between observed and expected frequencies of coexistence. We also assessed outcomes individually for each of the three parasite combinations: statistically significant deviations from expected were inferred when observed frequencies of outcomes (coexistence; single parasite 'winning') fell out of the range of the predicted distribution (see Gurney et al. 2017 for a similar procedure). Note that repeating the initial random pairings and bootstrap procedure for the *Obtusa*/*Undulata* combination does not change the results (Supplementary material Appendix 2 Fig A2c).

Finally, we tested whether observed frequencies of coinfection and of total infection prevalence differed from expected (Supplementary material Appendix 2). To be conservative, we chose the expected data set with the lowest frequency of predicted coexistence (and thus with the lowest expected frequency of coinfection). To compare expected and observed values, we used Fisher exact, Spearman rank and paired t-tests. All statistical analysis was performed using JMP 13.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.fg812n7> (Duncan et al. 2018).

Results

Single inoculations: persistence and prevalence

Three weeks post single inoculation, infection was detected in 96% of populations (68/71; one population was lost). The three *Holospora* parasites (*Caryophila*, *Undulata*, *Obtusa*) differed strongly in their infection prevalences (significant parasite effect, Supplementary material Appendix 2 Table A1). Near complete infection (>90%) was observed for *Caryophila* in all microcosm populations, whereas *Undulata* infected all populations, but typically only ca 30% of the individuals per population; *Obtusa* was not detected in three of the 24 populations and infected less than 15% of the individuals on average (Fig. 2, left panel). Each parasite was most infective on different host genetic backgrounds, even though rank order differences for overall infection levels were consistent across the three host genotypes (significant parasite × host genotype interaction; p = 0.0002; Supplementary material Appendix 2 Table A1).

Double inoculations

Parasite persistence and coexistence

Parasite persistence was observed in 71 of the 72 doubly inoculated populations. However, parasite coexistence was much less frequent than expected under independent action, with far more populations than expected being infected by only one parasite. Our simulated data sets predicted coexistence in a minimum of 63% of the populations (45 out of 72 populations, i.e. the sum of the minima of the ranges

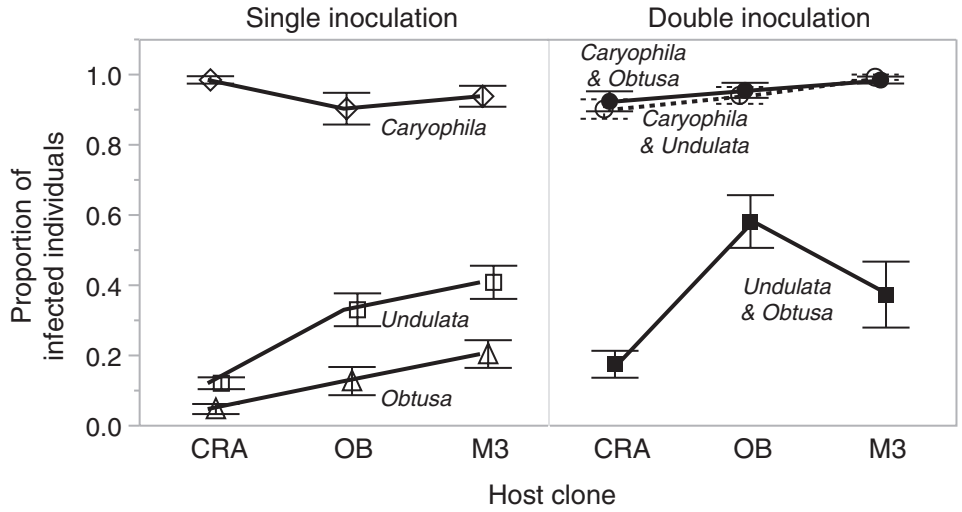

Figure 2. Mean (± SE) proportion of *Paramecium caudatum* cells infected by *Holospora caryophila, H. undulata or H. obtusa* in single inoculation treatments (left panel), and in double inoculation treatments of these parasites (right panel). Infection prevalences in double inoculations are based on the total number of cells infected by both or either parasite. Single and double inoculations were performed in microcosm populations of three *P. caudatum* clones (CRA, OB and M3).

for the expected values of coexistence in Fig. 3: 20 + 14 + 11), whereas the observed value was only 14% (10/72; Fisher's exact test: $p = 0.0002$). As shown in Fig. 3, this overall lack of coexistence held for all three combinations of parasites. More detailed analysis did not reveal significant effects of host clone on the probability of parasite coexistence (main effect and interaction with parasite combination: all $p > 0.26$; Supplementary material Appendix 2 Table A2). We note, however, that in one out of the nine cases (*Undulata* and *Obtusa* combination on host clone CRA) the observed frequency of coexistence was within the expected range (Supplementary material Appendix 2 Fig. A2c).

The probability to be the persisting 'winner' parasite followed a clear hierarchy, which reflected the order of infection success in the single inoculations. Thus the most infectious parasite, *Caryophila,* was maintained in all doubly inoculated populations, excluding in most cases (22/24) *Undulata* or (21/24) *Obtusa* (Fig. 3, left and middle panels). In *Undulata*/*Obtusa* double inoculations, *Undulata* was the single remaining parasite in 15 populations, compared to only three cases with *Obtusa* being 'the winner' (Fig. 3, right panel). Breaking down these 18 'wins' separately for each host clone (Supplementary material Appendix 2 Fig. A2c) indicates that at least one third fall within the expected range of outcomes. This may reflect the expected occasional non-establishment of *Obtusa* (3/24 = 12.5% absence in single infections) or the non-detection of the second parasite due to low infection prevalence.

Parasite prevalence.

We found the near-complete absence of coinfection. Only 2 of the 1098 infected cells inspected were simultaneously coinfected by both parasites (one *Caryophila*/*Undulata* and one *Undulata*/*Obtusa* coinfection). This is significantly fewer than the minimum number of 151 coinfections predicted from our simulated data (Fisher's exact test: $p < 0.0001$).

The absence of coinfection did not seem to affect total infection prevalence in the populations (the sum of all singly infected individuals with either parasite and coinfected cells). Overall, expected and observed values of total infection prevalences were highly correlated ($r = 0.84$, $n = 71$, $p < 0.0001$; Fig. 4) and did not significantly differ from one another ($t_{70} = 0.6$, $p > 0.5$). This general trend was largely confirmed by more detailed analysis, including parasite combination and host clone identity (Supplementary material Appendix 2 Table A3, Fig. A3).

Discussion

Our microcosm experiment revealed evidence for strong competition between different species of *Holospora* parasites at both the individual and population level. Indeed, very few coinfected host cells were observed and population-level parasite species coexistence was much less frequent than expected. The order of who 'won' this interspecific competition mirrored the infection success of the three parasites in single inoculations, with higher single infection prevalence being associated with higher exclusion capacity in double inoculations. These patterns resemble 'super-infection' scenarios employed by theoretical models, which assume within-host competition hierarchies and parasite exclusion (Nowak and May 1994).

Our results corroborate previous isolated observations in this system (Fokin et al. 2004, Görtz and Fokin 2009), showing that various endosymbionts can coinfect individual *Paramecium*, but in laboratory cultures typically one parasite takes over (Görtz and Fokin 2009). Temporary coexistence of different *Holospora* species and subsequent take-over by one

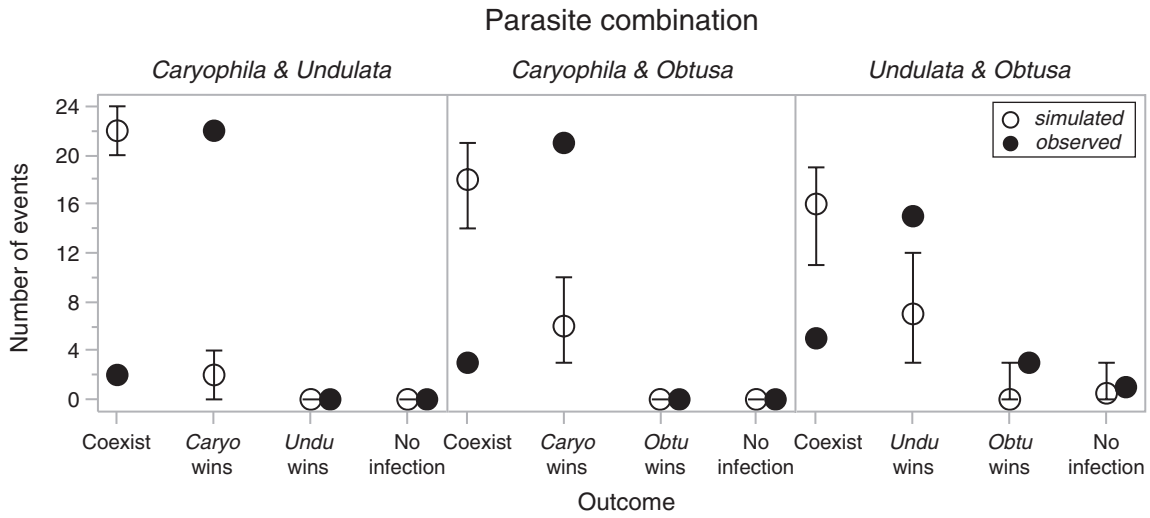

Figure 3. Frequency of expected (open circles) and observed (filled circles) infection outcomes in three double inoculation treatments of *Holospora caryophila*, *H. undulata* and *H. obtusa*, performed in experimental microcosms of *Paramecium caudatum*. Open circles correspond to the median number of expected outcomes and error bars to the range. Outcomes were: coexistence of parasites, detection of only one parasite ('wins'), or absence of any infection ('none'). In the majority of cases, when parasites coexisted, they resided in different host cells within a microcosm; coinfected cells were extremely rare. Expected and observed numbers add up to 24 in each panel, the number of replicate microcosms inoculated with each parasite combination. The calculation of the distribution of expected frequencies was based on infection success of each parasite in single inoculation treatments, carried out for 100 bootstrap runs (see Supplementary material Appendix 2 for details). A significant deviation from expected is inferred when observed values do not overlap with the distribution of the 100 expected values.

species is also documented for natural populations (e.g. *H. obtusa* replacing *H. elegans*; H. D. Görtz pers. comm.). Thus, our microcosm experiment seems to capture realistic aspects of processes of competitive exclusion of these parasites.

Patterns of competitive exclusion

Fokin et al. (2004) reported considerable levels of coinfection by the three parasites used in our study, following the first 48h after inoculation (Fokin et al. 2004). In contrast, three weeks post inoculation, we found that coinfected cells were practically absent (<<1%) and coexistence of parasites in the same population was much less frequent (15%) than expected (60%). Importantly, we generally observed the same parasites winning the competition across independent population replicates and for different genetic backgrounds of the host population. These results have two main implications. First, host (population) take-over mostly followed non-random rules, which parallel observed differences in infection success, with *Caryophila* >> *Undulata* > *Obtusa* (Fig. 2, left panel). Qualitatively, this translates into the quasi-deterministic success of *Caryophila*, whereas interactions between *Undulata* and *Obtusa* showed some element of stochasticity, as both were capable of monopolising populations.

Second, different mechanisms, within- and between hosts, may contribute to the observed population-level exclusion. As *Caryophila* infected almost 100% of the hosts, population-level outcomes are likely linked to the fate of cells coinfected with *Undulata* or *Obtusa*. In contrast, coinfection dynamics may be less important in the interaction between *Undulata* and *Obtusa*, as both parasites have relatively low infection success on their own (30% and 15%, respectively), which should lead to a low frequency of coinfected hosts (< 5%).

Mechanisms of population take-over

Population takeover by the *Caryophila* parasite can be explained by the loss or the absence of coinfected cells. One possibility is that coinfection with two parasites has a strong fitness disadvantage (Griffiths et al. 2011, Lass et al. 2012, Susi et al. 2015a), and thus doubly infected individuals may have been outcompeted by hosts harbouring single infections of *Caryophila*. This is conceivable, as *Caryophila* infection can even increase *Paramecium* growth rate over that of uninfected individuals (Bella et al. 2016; see additional data Supplementary material Appendix 2 Fig. A4). It would also explain the long-term maintenance of extremely high infection prevalences in our populations. In contrast, both *Undulata* and *Obtusa* typically reduce host division and survival (Restif and Kaltz 2006, Fujishima 2009) and infection prevalences remain at intermediate levels, as was found here (see also Duncan et al. 2011, 2013).

Alternatively, *Caryophila* takeover may be the result of within-host competition via depletion of a common resource or pre-emption of space (Ulrich and Schmid-Hempel 2012, Griffiths et al. 2014, Abkallo et al. 2015). *Holospora* parasites likely experience competition for space, as they are relatively large compared to the size of the host compartments they infect. Thus, *Caryophila* may outnumber *Obtusa* in the

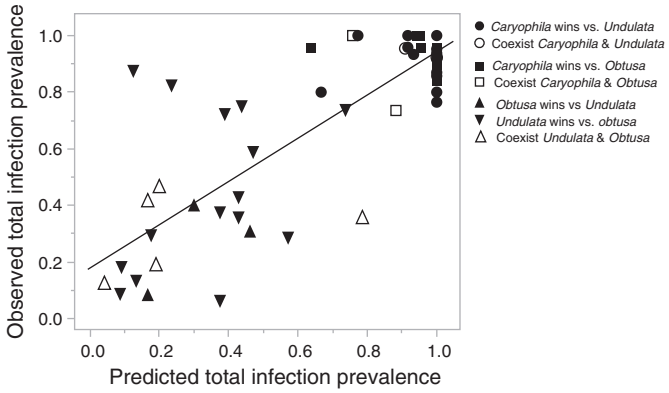

Figure 4. Correlation of expected and observed infection prevalences in microcosm populations coinoculated with *Caryophila* and *Undulata* (circles), *Caryophila* and *Obtusa* (squares) and *Obtusa* and *Undulata* (triangles). Open symbols denote populations where both parasites coexisted and closed symbols where only one persisted. Infection prevalence is the proportion of infected individuals in a population; it was calculated from the sum of all infected individuals in the population (combined for both parasites, in cases of coexistence). The line represents a linear regression ($r^2 = 0.71$).

macronucleus through faster multiplication of reproductive forms, possibly over successive rounds of host divisions, during which reproductive forms are vertically transmitted to host daughter nuclei. Within-host replication may also mediate competition across the macro- and micronucleus compartments. Continued within-host replication can lead to hypertrophic nuclei that fill the entire host cell (Fokin and Görtz 2009). Thus, a faster swelling *Caryophila*-infected macronucleus may prevent the expansion of an *Undulata*-infected micronucleus and thereby suppress this competitor through pre-emption of space.

Another extreme form of pre-emption might be that resident parasites protect hosts against additional infection, e.g. through the activation of the host defence system (Brown and Grenfell 2001, Greiner et al. 2009). There is some evidence that *Holospora* infections modify host defence or behaviour, thereby reducing the uptake of additional parasites or blocking their transfer to the nucleus (Fels et al. 2008, Fokin and Görtz 2009). However, this explanation would require that *Caryophila* parasites consistently arrive first in the host to activate the blocking against *Undulata* and *Obtusa*, e.g. if they have a shorter travel time from the vacuole to the macronucleus or start within-host replication more rapidly.

As coinfections were expected to be rare, population take-over in the *Undulata* and *Obtusa* treatment cannot be explained by within-host competition or the loss of coinfected cells. Direct (chemical) warfare between the two parasites in the inoculum is unlikely, as infectious forms appear to be metabolically inert during the transmission period and only start differentiation once they are in the micronucleus (Fujishima 2009). There is also no evidence for direct interactions between singly infected hosts, e.g. via toxins, as is known for other *Paramecium* symbionts (Schrallhammer and Schweikert 2009). We speculate that interactions might have occurred during the initial infection phase. Possibly, in the presence of multiple parasites in the inoculum, *Paramecium* are more likely to mount avoidance reactions, for example by reducing filtering rate and therefore contact with infectious forms (O. Kaltz unpubl.; see also Fels et al. 2008). If this reduces host availability, then the already less infectious *Obtusa* parasite may only be able to infect very few, or no, cells in the initial cohort of available *Paramecium*, and thus subsequently fail to persist in the population. This may represent an example of an Allee or drift effect, leading to the failure of an epidemic, even though a parasite has an intrinsically positive R_0 (Hartfield and Alizon 2013). As these two parasites are more similar in their general infectivity, such a stochastic component would explain why outcomes were less consistent than for co-inoculations with the highly infectious *Caryophila*.

Experimental tests of these different hypotheses would require more detailed investigations, including sequential inoculation protocols, time series of infection dynamics (in particular soon after inoculation) or fitness assays of doubly versus singly infected hosts, which was beyond the scope of the present study.

Relation to standard super-infection scenarios

Our observed competition hierarchies and complete host-takeover are consistent with classic 'super-infection' scenarios, used in theoretical models where higher within-host growth is accompanied with competitive exclusion (Nowak and May 1994, but see Sofonea et al. 2016). Mutual competitive exclusion of phage has been demonstrated in bacteria host populations (Delbrück 1945), competing fungal endophytes (Wille et al. 2002), virus strains within their host plants (Syller and Grupa 2016) or malaria parasites in mice (de Roode et al. 2004, Abkallo et al. 2015). However, only the malaria parasites show a linear hierarchy for strain exclusion (de Roode et al. 2004, Abkallo et al. 2015), the outcomes in the other examples being dependent on who arrived first (Delbrück 1945, Wille et al. 2002, Syller and Grupa 2016).

Contrary to standard assumptions of super-infection models (Nowak and May 1994), the competitive hierarchy in our system is probably not related to virulence, as the competitively superior *Caryophila* is less virulent than *Obtusa* or *Undulata* (Bella et al. 2016) (Supplementary material Appendix 2 Fig. A4*)*. Similarly, avirulent strains of *Plasmodium yoelli* were able to competitively exclude more virulent strains (Abkallo et al. 2015), in contrast to *P. chabaudi* (de Roode et al. 2004). Indeed, as highlighted by Sofonea et al. (2016), the traditional super-infection versus coinfection dichotomy ignores many possible outcomes, one of which is that selection through competitive exclusion may be accompanied by lower virulence (Sofonea et al. 2016), as observed in our experiment.

Effect of host genetic background on competition outcomes

There was some variation in levels of persistence and prevalence in coinoculated populations due to host genotype, but no change in the rank order of infection. This may be surprising considering the large number of studies showing interactions between host and parasite genotypes, often with crossing reaction norms for infectivity and resistance related traits (Lambrechts et al. 2005, de Roode and Altizer 2010), including for *Paramecium–Holospora* interactions (Adiba et al. 2010, Duncan et al. 2010, Fellous et al. 2012, Bella et al. 2016). Furthermore, both parasite and host genotype have been shown to change which parasite has higher fitness in a coinfection scenario (Louhi et al. 2015, Klemme et al. 2016, Orsucci et al. 2016). Nevertheless, other studies show host genotype does not necessarily change the rank order of parasite fitness in coinfections, even when genotype impacts individual parasite traits (Susi et al. 2015b).

Implications

The occurrence of competitive hierarchies that are consistent across different host genetic backgrounds has important implications for public health and parasite management. Indeed, epidemiological forecasting would be easier to model across a homogeneous host population with only susceptible, infected and recovered hosts. Similarly, if an avirulent parasite always excludes a more virulent parasite, this could be used for curative treatment, or as a prophylactic to prevent invasion. For instance, *Plasmodium* was used to cure people from the later stages of syphilis in the early 20th century (Bynum 2010). However, the more stochastic nature of interactions between *Obtusa* and *Undulata* may be a more realistic scenario, where one parasite tends to outcompete the other, but not always. This parallels with predictable epidemiological patterns for measles enabling the production of accurate projection models, which are not possible for whooping cough which exhibits a more stochastic epidemiology (Rohani and King 2010). Caution should also be exercised as outcomes may still change across different environmental conditions. For example, food availability has been found to impact parasite transmission traits in coinfections across a number of systems (Hodgson et al. 2004, Budischak et al. 2015, Duncan et al. 2015).

Conclusions

This microcosm study shows that parasites can repeatedly respond to different competitor species in the same way over multiple host generations and with experimentally unconstrained transmission dynamics. Indeed, we show a clear competitive hierarchy among the three parasite species, *Holospora caryophila, H. undulata* and *H. obtusa*, reflecting their infection success in single inoculation treatments. Our results are consistent with super-infection models, whereby one parasite always wins through competitive exclusion of another. However, different within- and between-host mechanisms may have produced exclusion, and we have good evidence that competitive superiority here is associated with lower, rather than higher, virulence. On the other hand, our observations of a mostly fixed competitive hierarchy and a relatively weak impact of host genetic background suggest that simplifying assumptions of theoretical models may not necessarily be unrealistic.

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Supplementary material (available online as Appendix oik-05280 at www.oikosjournal.org/appendix/oik-05280>). Appendix 1–2.