



# Invading the Occupied Niche: How a Parasitic Copepod of Introduced Oysters Can Expel a Congener From Native Mussels

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In species introductions, non-native species are often confronted with new niches occupied by more specialized natives, and for introduced parasites this conflict can be amplified because they also face novel hosts. Despite these obstacles, invasions of introduced parasites occur frequently, but the mechanisms that facilitate parasite invasion success are only rarely explored. Here, we investigated how the parasitic copepod *Mytilicola orientalis*, that recently spilled over from its principal host - the Pacific oyster *Crassostrea gigas*, managed to invade the niche of blue mussel *Mytilus edulis* intestines, which is densely occupied by its specialist congener, *Mytilicola intestinalis*. From field observations demonstrating invasion dynamics in nature, we designed a series of experiments addressing potential mechanisms facilitating a successful occupation of the new niche. As expected the specialist *M. intestinalis* can only infect mussel hosts, but displayed higher infection success there than *M. orientalis* in both principal host species combined. In the absence of direct competitive interactions *M. orientalis* compensated its lower infection success (1) by recurrent spill-over from its high-fitness reservoir oyster host, and (2) by active aggregation interference enhancing its own mating success while limiting that of *M. intestinalis*. The introduced parasite could thus avoid direct competition by changing its own epidemiology and indirectly decreasing the reproductive success of its competitor in the new host. Such mechanisms outside of direct competition have seldom been considered, but are crucial to understand invasion success, parasite host range and community assembly in the context of species introductions.

**Keywords:** biological invasion, epidemiology, host choice, macroparasite, mollusk, spill-over, Wadden Sea

## 1 INTRODUCTION

Fuelled by global shipping and aquaculture activities marine ecosystems experienced an increasing number of species introductions in recent years (Bailey et al., 2020). When becoming invasive, introduced species can have a series of economic (Cuthbert et al., 2021) and foremost ecological consequences (Anton et al., 2019). In comparison, invasive parasites and their effect are investigated

only rarely (Bailey et al., 2020). Recently several studies investigated the effect of invasive parasites and showed that parasites can have profound effects when introduced parasites cause disease (Bouwmeester et al., 2021) and affect invasion processes of free living species (Goedknecht et al., 2016a). Invasive parasites face a new environment that fights back – the host. Within the host they will often encounter established native parasites further intensifying selective pressures by competition exposing invaders to series of disadvantages. First of all, the newly introduced parasite will be rare, which alone can prevent establishment (Quigley et al., 2018). Furthermore, the introduced parasite will by definition be less adapted to the new host than the established parasite that has shared some coevolutionary history with the hosts (Feis et al., 2016; Blakeslee et al., 2020). Despite these unfavorable starting conditions, there are many examples of parasites that defy the odds and successfully invade occupied host niches (Goedknecht et al., 2016a). However, hardly anything is known about the mechanistic basis of species interactions and competition when introduced parasites meet native ones.

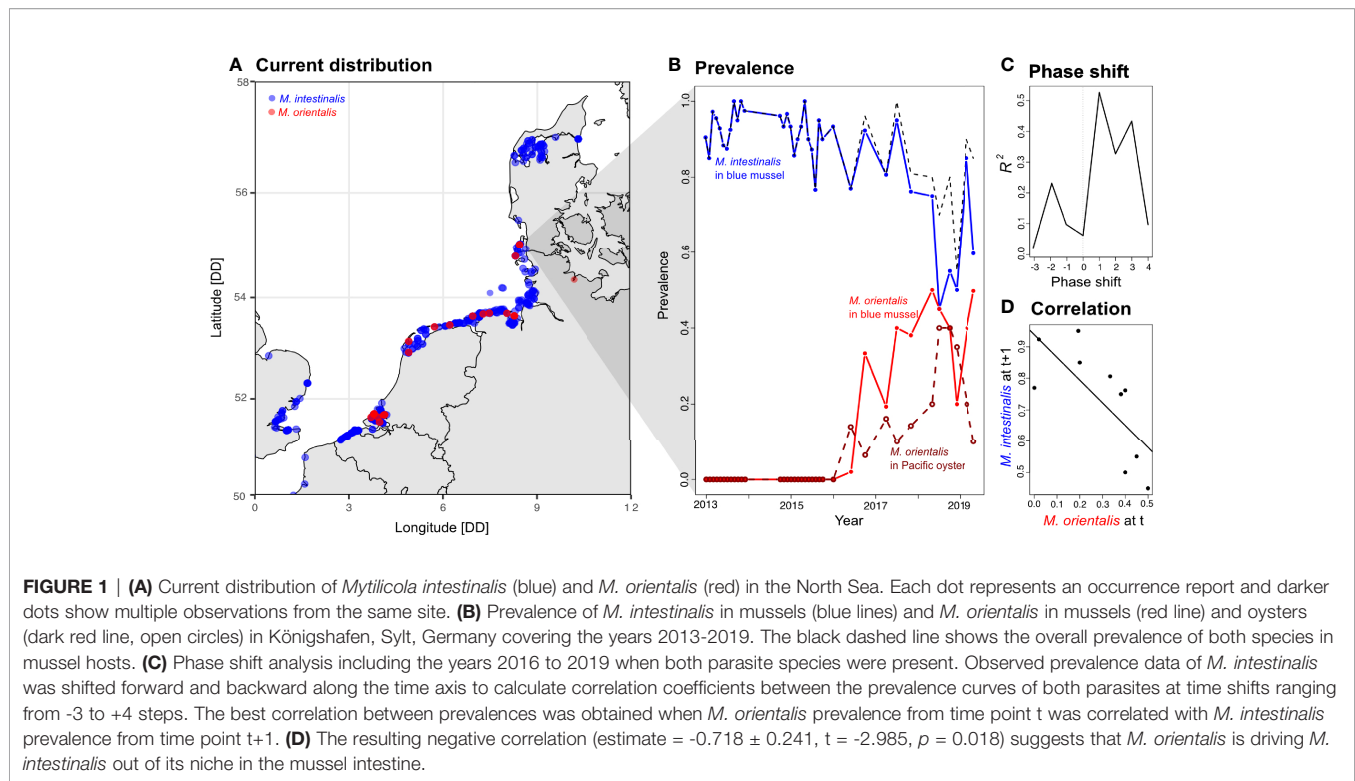
In general, host-parasite coupled to parasite-parasite coevolution could lead to the use of separate niches within the same host to avoid competition (Holmes, 1973). Consequently, research efforts have focused on competitive interactions to explain parasite community assembly and niche use within hosts (Mideo, 2009). In this context studies found superiority effects in simultaneous co-infections (Leung and Poulin, 2011), often finding that more generalistic species with a wider host range have a greater sensitivity to competition than specialists representing a cost of generalism (Dawson et al., 2000; Okabe et al., 2012). Similarly, priority effects in sequential infections revealed either antagonistic (Hoverman et al., 2013), facilitative (Halliday et al., 2020) but also no detectable interaction (Zilio and Koella, 2020) between prior and later infections. These different outcomes of competitive interaction among parasites can be attributed to their shared coevolutionary history, and therefore do not offer a direct comparison of the fitness landscapes parasites face when initially confronting each other. In recent biological invasions these conditions can be tested (Feis et al., 2016; Blakeslee et al., 2020). Introduced parasites can be forced to invade new niches (Frankel et al., 2015; Tepolt et al., 2020) that will often be occupied by native parasites (Goedknecht et al., 2016a) making them ideal systems to investigate the species interactions at the onset of competition.

Here, we utilize the recent invasion of the parasitic copepod *Mytilicola orientalis* leading to novel interactions with its congener *Mytilicola intestinalis*. *M. orientalis* was co-introduced with Pacific oysters *Crassostrea gigas* around the globe and also invaded the European Wadden Sea in the last decades (Feis et al., 2019), where it is displacing *M. intestinalis* in its specialized niche (**Figure 1A**). Spatially both parasites use the same niche and infest intestines of mollusks (Goedknecht et al., 2018b), where they attach with hooklike structures to the intestinal wall (Figueras et al., 1991; Bignell et al., 2008). Parasite attachment causes lesions and inflammations (Watermann et al., 2008) that invoke a costly immune response (Santarem et al., 1994) and can lead to

reduced body condition (Goedknecht et al., 2018a). Relative to their intestinal habitat both parasites can reach considerable sizes (up to 12 mm (Goedknecht et al., 2018a)). Their large size and high infection prevalences and intensities spatially restrict niche availability, leading to competition between individuals (Feis et al., 2016). In the Wadden Sea, *Mytilicola intestinalis* is found exclusively in blue mussels *Mytilus edulis* (Elsner et al., 2011; Goedknecht, 2017), creating a narrow realized host range characteristic for specialist parasites. *Mytilicola orientalis*, on the other hand, has been globally distributed with aquaculture of Pacific oysters (Feis et al., 2019), from which it repeatedly spilled over into native *Mytilus* populations and rarely into other mollusks (Stock, 1993; Goedknecht et al., 2016b), thus displaying a wider host range than *M. intestinalis*. Both species reproduce sexually in the intestine of their hosts, and only parasites infecting a host that is already infected by another parasite of the opposite sex will get the chance to mate. Remaining the only infection in a host will inevitably lead to no reproduction, potentially creating allele effects that can decelerate population growth (Regoes et al., 2002; Bercé et al., 2007). Therefore, aggregation within hosts is important for sexually reproducing parasites like *Mytilicola* and aggregation interference between competitors can influence population growth.

In regions where both species already occur in sympatry (e.g. Texel in the southern Wadden Sea, the Netherlands) a decline of *M. intestinalis* from 60% in 1976 (Drinkwaard, 1993) to 13% in 2013 (Goedknecht, 2017) was observed coinciding with the arrival of *M. orientalis*. This might suggest that the generalist newcomer drove out the established specialist. However, it is uncertain if the generalist *M. orientalis* took over the specialist's niche by direct competitive interactions, or whether abundance of *M. intestinalis* declined for other reasons that opened up its niche in the intestines of mussels.

Here, we now focus on the ongoing invasion of *M. orientalis* along the edge of its current distribution in the northern European Wadden Sea (Sylt, Germany, **Figure 1A**), where the recent arrival of *M. orientalis* in 2016 led to a delayed decline of *M. intestinalis* from very high (>90%) to considerably lower prevalences (<60%) over the time course of a few years (**Figure 1B**). Against this backdrop of a successful parasite invasion, we designed a series of experiments with the two ecologically relevant hosts *M. edulis* and *C. gigas* to reveal potential mechanisms that might give *M. orientalis* a competitive edge over *M. intestinalis* in the shared host, *M. edulis*. In detail, we assessed the competitive balance between the two species by testing superiority (i.e. competitive advantage in simultaneous infections) and priority effects (i.e. competitive advantage arising from the sequence of infections). To determine whether trade-offs in infectivity establish a cost of generalism, we investigated the infectivity of both parasites in both host species. We also asked whether recurrent spill-over of high fitness propagules could drive *M. orientalis* epidemiology by testing whether infectivity of *M. orientalis* was higher when originating from its reservoir hosts (Pacific oyster) or its newly acquired hosts (blue mussel). Finally, we tested if infection behavior in choice experiments is affected by the presence of



previous con- and hetero-specific infections. Preference for previously infected individuals could then lead to aggregated distributions, which particularly important in initial stages of an invasion when the introduced species is still rare. Our experiments thus combine multiple mechanistic explanations that extend the scope for our understanding of how restricted and densely occupied niches can be invaded.

## 2 MATERIALS AND METHODS

### 2.1 Monitoring the Ongoing Invasion of *M. orientalis*

To document the ongoing invasion of *Mytilicola orientalis* into the niche of *M. intestinalis* in the wild, we collected Pacific oysters *Crassostrea gigas* and blue mussels *Mytilus edulis* in the “oyssel reef” (a multilayered, mixed bed of mussels and oysters, *sensu* (Reise et al., 2017b)) in Königshafen, Sylt, Germany (55°02'17" N, 08°26'32" E) between 2013 and 2019. *Mytilicola orientalis* was absent from this location in 2008 (Elsner et al., 2011). For each time point, 20-30 individuals of both species were dissected under a stereomicroscope to count *Mytilicola* infections in the digestive tracts based on morphological species determination (Goedknecht et al., 2018b). We calculated prevalence (i.e. the proportion of hosts infected in a sample) and intensity (i.e. mean number of parasites in infected hosts within a sample) of both parasite species for each host species and time point.

To test the causal direction between the prevalences of both parasites, we performed a phase-shift analysis for the years between 2016 and 2019, when both parasites were present.

Hereto, we shifted the observed prevalence data of *M. intestinalis* forward and backward along the time axis and calculated correlation coefficients between both prevalence curves at time shifts ranging from -3 to +4 sampling intervals. While this procedure cannot produce causality by itself, only past events can theoretically influence future observations. In other words, if the best correlations between the prevalence curves is observed at positive shift values (i.e. *M. intestinalis* prevalence is pushed into the future), current observations of *M. orientalis* correlate best with future observations of *M. intestinalis* suggesting that *M. orientalis* prevalence drives *M. intestinalis* prevalence. On the other hand, negative shift values giving the best correlation might rather suggest that *M. intestinalis* declines for unknown reasons and *M. orientalis* is taking advantage of the emptying niche in the intestine of mussels.

From the abundance data (i.e. the number of parasites found in each host) we also calculated parasite aggregation and the likelihood of finding a mate in an infected host. Aggregation was expressed as the variance-to-mean ratio calculated from the number of parasites found in each host for each sampling time point in all three host-parasite combinations (*M. intestinalis* in mussels, *M. orientalis* in mussels and *M. orientalis* in oysters). A variance-to-mean ratio of 1 indicates that data is distributed according to a Poisson distribution. Higher values indicate a higher degree of aggregation resulting from a clumped distribution with only few hosts carrying many parasites while many hosts show low infection intensities or no infection at all (Barbour and Pugliese, 2000). The likelihood of finding a mate, i.e. meeting a conspecific of the opposite sex, was calculated from binomial proportions of males and females assuming the simplest

case of a 1:1 sex ratio based on the collected abundance data. In other words, if a parasite ends up in a host with only one other parasite, it has a 50% chance that the other specimen is of the opposite sex, whereas there is already a 96% chance to encounter the opposite sex when 5 parasites were already present.

## 2.2 Infection Experiments

### 2.2.1 General Infection Procedure

All experimental infections followed previously established protocols (Feis et al., 2016; Demann and Wegner, 2019). In short, uninfected mussel hosts (3.5–5 cm) from Sylt were obtained either by insecticide treatment (Blateau et al., 1992), only in simultaneous infections) or by sampling in populations with naturally low prevalence <10% (west shore breakers (Demann and Wegner, 2019)). For infection, we collected egg sacs from individual gravid female parasites, and we infected hosts in individual tanks by adding 24 copepodites. Copepodites not ingested by the host were counted after 24 h to calculate the exposure dose for each host. During the experiments mussels were kept individually in a flow-through system with algal food supply. We dissected hosts after 51–80 days and counted parasites in the intestine. Parasite species were identified by morphological characteristics or species-specific genetic polymorphisms (Goedknecht et al., 2018b), and infection rates were calculated as the proportion of successful infections from all ingested copepodites. We also included control groups in all experiments to check for background infections not originating from our controlled infections. Control mussels were treated the same way but were not exposed to copepodites. We only observed background infections in the preference experiment and removed those hosts as well as control hosts from infection rate analyses.

### 2.2.2 Simultaneous and Sequential Infections

To test the competitive balance between the two parasite species we assessed their infectivity in the shared host by conducting two co-infection experiments. Simultaneous infections were used to test superiority effects and sequential infections were used to establish priority effects (Figure 2). Both sets used a substitution design. In simultaneous infections we added 24 copepodites in the single species infections and mixes of 12 *M. intestinalis* and 12 *M. orientalis* copepodites for co-infections. This resulted in three experimental groups of 15 mussels for the simultaneous infections (n = 45). Infective copepodites of *M. intestinalis* were obtained from egg sacs collected in Oddewatt, Sylt, whereas we used egg sacs from Texel to obtain *M. orientalis* copepodites, as *M. orientalis* was not found on Sylt at the time both experiments were conducted (2014 and 2016, Figure 2). Infection rates were analyzed with binomial generalized linear mixed models (GLMM) using the R package *lme4*. The number of successful against failed infections from all ingested copepodites in each mussel was used as the binomial response. We recorded infection intensities for both parasites in co-infections separately and fitted “mussel ID” as a random intercept to account for the two infection intensities coming from the same host. We fitted “infection type” (single vs. co-infection) testing for differences in infection rates between single and co-infections, “parasite species” (*M. intestinalis* vs. *M. orientalis*) testing for differences in infection success

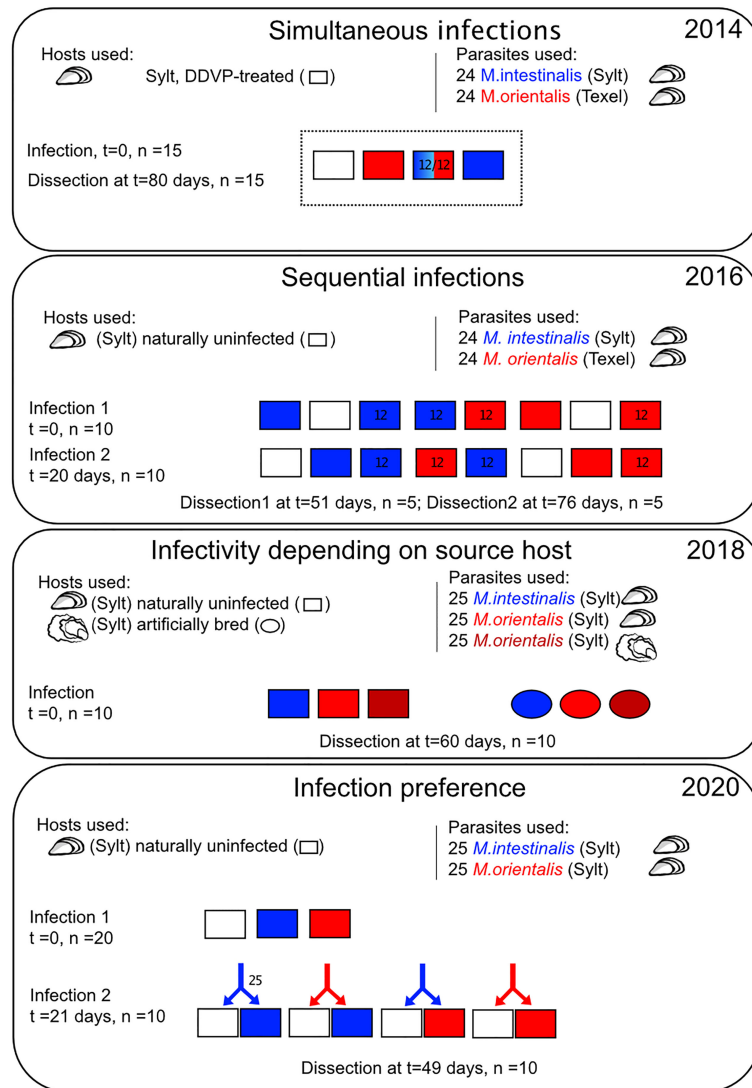
between both parasite species and the interaction term describing whether differences between single and co-infections were dependent on the focal parasite species. Pairwise differences between treatment combinations were tested by Tukey-Posthoc test implemented in the *multcomp* package (Hothorn et al., 2008).

For the sequential infections we used two rounds of infection separated by 20 days. To account for using different batches of copepodites between both infection rounds, we conducted single species infections with 24 copepodites of both parasites at both time points using a different set of hosts each time. The sequential infections consisted of mussels, which were either exposed twice (mixed infections) or once (single species infections) in all possible combinations resulting in 8 treatment groups of 10 mussels each (n = 80). We dissected the sequential infection experiment in two rounds of 5 individuals per treatment group to allow size differences between parasites to increase between first and second infections. Because there were no quantitative differences in infection rates between both time points, we analyzed the whole dataset together. We fitted a model similar to simultaneous infections for sequential infections. Next to the random factor of “mussel ID”, we fitted the fixed effects “parasite species” (*M. intestinalis* vs. *M. orientalis*), the modified three-level factor “infection type” (single infection in either the 1st or 2nd infection round vs. con-specific infection using the same species in both infection rounds vs. hetero-specific infection using the other parasite species in the 2nd round) and the additional factor “infection round” (1st infection round vs. 2nd infection round). These main effects tested for differences between the factor levels of each factor, while the three-way interaction between parasite species, infection type and infection round tested whether either parasite species reacted differently on the prior presence of the same or other parasite species.

### 2.2.3 Infectivity of Offspring Originating From Different Host Sources

This experiment was designed to test if *M. orientalis* is showing lower infection success in both of its host species consistent with a cost of generalism. Furthermore, we were also interested in whether mothers produce offspring of higher fitness/infectivity when they were isolated from oyster or mussel hosts, and whether the origin specifically influences infection rates in both host species. Therefore, we combined a source host treatment (*M. intestinalis* from mussels/*M. orientalis* from mussels/*M. orientalis* from oysters) with an infection host treatment (mussels/oysters) in a full factorial design, resulting in six treatment groups consisting of 10 hosts each (Figure 2).

Naturally uninfected mussels and Pacific oysters (1.5 year old lab bred broodstock) of shell lengths 4.0 – 4.6 cm were used as infection hosts. Mussels and oysters from the Oddewatt oysterreef also served as donors for *M. intestinalis* and *M. orientalis* copepodites. Infection rates were analyzed by binomial GLMs comparing the number of successful vs. failed infections as a function of “source host” (*M. intestinalis* vs. *M. orientalis* originating from mussels vs. *M. orientalis* originating from oysters) and “infection host” (mussel vs. oyster). The main effect “source host” tested if infection success depended on the host copepodites originated from, while the main effect “infection host”



**FIGURE 2** | Schematic representation of the experimental infections. Each panel shows one experimental setup and the year in which the experiment was conducted. Boxes represent batches of experimental mussel and ovals batches of oyster hosts with blue coding for infections by *M. intestinalis*, red for infections by *M. orientalis* originating from mussels, and dark red for *M. orientalis* originating from oysters. Batch size is given by the n for each experiment. The geographic source of hosts and parasites is shown in brackets and source hosts are shown by pictograms.

tested for differences between the host species used in the infection. The interaction between both terms would indicate that infection success was higher in specific combinations of source and infection hosts.

### 2.2.4 Parasite Infection Preference

This experiment aimed at testing whether *Mytilicola* species prefer to infect uninfected or previously infected hosts. Preference for hosts previously infected by conspecifics will lead to higher parasite aggregation within hosts, which can be equated to more opportunities to reproduce. We tested preference by offering a batch of 25 copepodites the choice of an experimentally infected and an uninfected host. To obtain infected hosts, we infected

naturally uninfected mussels of shell lengths 2.8 – 4.2 cm with either 25 *M. intestinalis* or 25 *M. orientalis* copepodites. Each mussel from this group was then paired with a size-matched uninfected mussel and both were maintained in separate containers in the flow-through system until the actual preference infection three weeks later. The two matching hosts were then placed in a single container and were exposed to either 25 *M. intestinalis* or 25 *M. orientalis* copepodites. As the primary infections stretched out over several weeks, we always made sure that we used the same batch of copepodites for one preference infection for matching *M. intestinalis* and *M. orientalis* primary infections conducted at the same time ( $\pm 2$  days). After 24 h, hosts were put back into their individual containers until the end of the

experiment seven weeks after the primary infection ( $n = 45$ ). Due to the paired design in each preference infection, we analyzed the data as the difference between infection rates of the host with primary infection minus the infection rate of the uninfected host. Positive values therefore reflect a preference for the infected host, while negative values show preference for the uninfected host. This preference score was then analyzed by a linear model, fitting the number of parasites from the first infection to control for dose dependency in the preference infection, the “parasite species” used for the second preference infection (*M. intestinalis* vs. *M. orientalis*) and a factor describing whether the “primary infection” used the same (con-specific) or the other parasite species (hetero-specific).

### 3 RESULTS

#### 3.1 Temporal Dynamics of *Mytilicola* Infections in the Field

During the period 2013 to 2016, *M. intestinalis* was the only *Mytilicola* species found in mussel intestines on the Oddewatt oyster reef (Figure 1B). With some seasonal fluctuations, *M. intestinalis* was found in high prevalence (between 77% and 100%) coupled to high mean infection intensities (between 4.2 and 10.4) reaching a maximal individual infection intensity of 42 parasites (Figure 1B). Multiple *M. orientalis* were found first in June 2016, when 2% of the mussels were infected. During the following years, *M. orientalis* increased in oysters as well as in mussels, while *M. intestinalis* prevalence steadily decreased and dipped below the prevalence of *M. orientalis* in mussels in 2018 (Figure 1B). Mean infection intensities of *M. orientalis* in mussels ranged between 1.5 and 3.1 and were somewhat lower than intensities in oyster, which ranged from 1.7 to 18.3 parasites per infected host. We found up to 46 *M. orientalis* specimen in a single oyster, but never found *M. intestinalis* in the 222 Pacific oysters we dissected. Our phase-shift analysis of prevalences in years where both parasite species were present (2016-2019) revealed that shifting *M. intestinalis* prevalences one sampling interval into the future (phase shift +1) gave the best correlation coefficient between both curves (Figure 1C). The significant negative correlation at this phase shift thus indicated that the prevalence of *M. intestinalis* was negatively affected by *M. orientalis* in mussels at the previous time point in field data (Figure 1D). This demonstrates the ongoing successful invasion of *M. orientalis* into the occupied niche of the mussel intestine and might indicate a competitive displacement of *M. intestinalis*.

The distribution of parasites found in each host also differed between the host-parasite combinations. Both parasites showed aggregated distributions with many uninfected and only few infected hosts with a high number of infections (Figure 3A). We nevertheless found significantly different degrees of aggregation (variance to mean ratio) between the different host-parasite combinations, with *M. orientalis* in oysters showing the highest degree of aggregation (Figure 3B). High aggregation values in *M. intestinalis* infecting mussels were only observed in the early years (2013-2016) with aggregation dropping substantially after

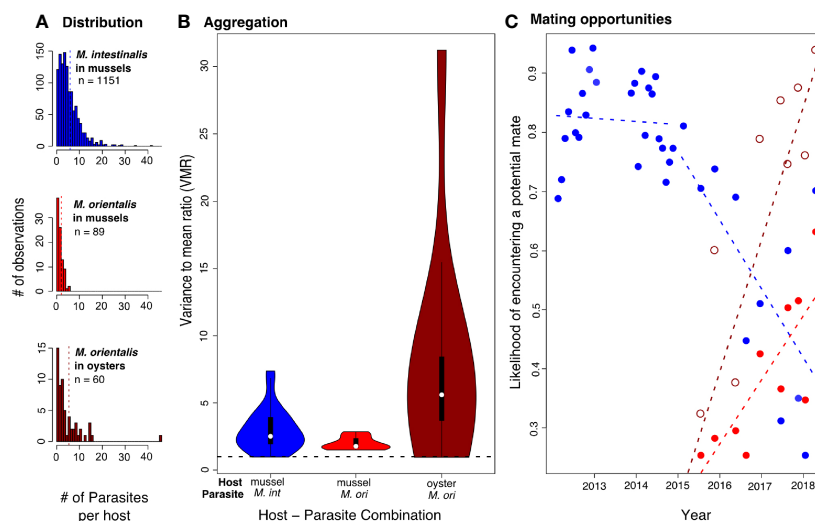
*M. orientalis* invaded. The lower aggregation values in the later years also lead to a significantly decreased likelihood of encountering a mate in an infected host for *M. intestinalis* after *M. orientalis* invaded (Figure 3C). For *M. orientalis*, on the other hand, mating opportunities significantly increased in mussels and reached the highest values in oysters although this increase was only marginally significant (Figure 3C).

#### 3.2 No Competitive Interaction in Simultaneous and Sequential Infections

In both simultaneous and sequential infection experiments, the specialist *M. intestinalis* had a higher infection rate than the generalist *M. orientalis* in the shared mussel host (Figure 4). During the simultaneous infection experiment eight mussels died (co-infection: 2, *M. intestinalis*: 2, *M. orientalis*: 4) and we observed a mean infection rate of  $0.486 \pm 0.054$  for single species infections of *M. intestinalis* and  $0.164 \pm 0.047$  for *M. orientalis*. No significant difference could be observed when comparing the single parasite infection rates to the parasite specific infection rates in the co-infection treatment of  $0.506 \pm 0.076$  for *M. intestinalis* and  $0.160 \pm 0.043$  for *M. orientalis* (Figure 4A and Table 1A), indicating that the presence of the other species did not influence infection success during the infection process. In sequential infections we observed a significant decline in infection rates between the first and second round of infections. This decline was mainly driven by low infection success of the second round of *M. intestinalis* infections, which was not observed in *M. orientalis*. In fact, pairwise comparisons revealed that the majority (7 out of 10) of significant differences were found between parasite species, while the only significant differences within parasite species were found between round 1 and round 2 infections with *M. intestinalis*. More importantly and resembling results obtained in simultaneous infections, we could not find significant differences between single infections and their corresponding infection rates in hetero-specific sequential infections (Figure 4B and Table 1B, *M. intestinalis* 1st infection round: single =  $0.687 \pm 0.049$  vs. hetero-specific =  $0.703 \pm 0.079$ , 2nd infection: single =  $0.348 \pm 0.087$  vs. hetero-specific =  $0.404 \pm 0.082$ , *M. orientalis* 1st infection: single =  $0.423 \pm 0.084$  vs. hetero-specific =  $0.215 \pm 0.046$ , 2nd infection: single =  $0.266 \pm 0.072$  vs. hetero-specific =  $0.313 \pm 0.099$ , all Tukey PostHoc comparisons  $p > 0.9$ ). These results indicate that direct competition between *M. intestinalis* and *M. orientalis* did not affect infection success, neither by superiority in simultaneous infections, nor by priority in sequential infections where one species was given a head start over the other.

#### 3.3 *M. orientalis* From Oysters Have Higher Infectivity

Compared to *M. intestinalis* infection rates were significantly lower for *M. orientalis* when infecting its newly acquired host *M. edulis* (estimate =  $-2.680 \pm 0.203$ ,  $z = -13.192$ ,  $p < 0.001$ ) but also when infecting its reservoir host *C. gigas* (estimate =  $-2.558 \pm 0.206$ ,  $z = -12.409$ ,  $p < 0.001$ ). Even when combining infection rates for both hosts median infection rates of *M. orientalis* (0.082) was only 15% of the median infection rate of *M. intestinalis*



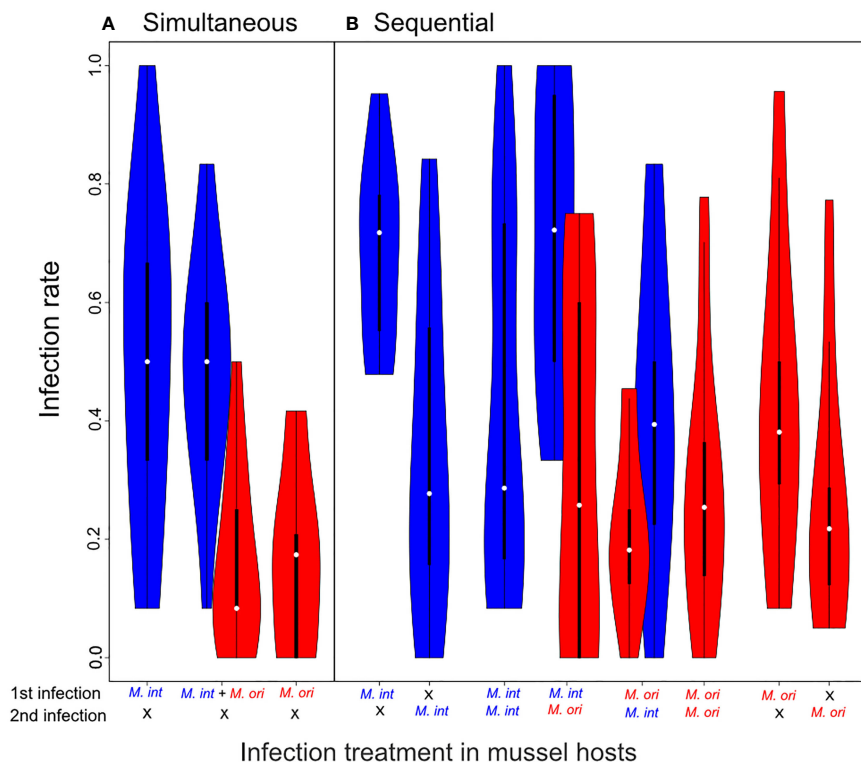
**FIGURE 3 | (A)** Distribution of number of parasites per host for *M. intestinalis* (*M. int.*, blue) and *M. orientalis* (*M. ori.*, red, dark red) over all years of the field survey. Dashed vertical lines show the mean. **(B)** Aggregation of parasites expressed as the variance to mean ratio (VMR) for all years of the field survey. Variance and means were calculated from infection data of each parasite species found in all hosts dissected at each sampling time point. Violins show the range of the observed data with violin width representing the frequency distribution of values. Box and whiskers give the 50 and 95 percentile, and white dots within boxes show the median. Aggregation was significantly stronger for *M. orientalis* in oysters than for *M. intestinalis* in mussels (estimate =  $4.570 \pm 1.303$ ,  $t = 3.507$ ,  $p < 0.001$ ) but did not differ between the parasite species when infecting mussels (estimate =  $-0.880 \pm 1.303$ ,  $t = -0.675$ ,  $p = 0.502$ ). **(C)** Likelihood of encountering a mate in an infected host on a given sampling event assuming the simplest case of a 1:1 sex ratio. In years without (2012–2016) *Mytilicola orientalis* mating opportunities for *Mytilicola intestinalis* (blue dots) showed no significant trend ( $R^2 = 0.005$ , estimate =  $-0.000 \pm 0.001$ ,  $t = -0.34$ ,  $p = 0.737$ ). However, after the appearance of *M. orientalis* in 2016 a significant decline over the sampling period was observed ( $R^2 = 0.421$ , estimate =  $-0.010 \pm 0.004$ ,  $t = -2.559$ ,  $p = 0.031$ ), while *M. orientalis*' chances of encountering a potential mate in a given host increased in both hosts (mussel:  $R^2 = 0.576$ , estimate =  $0.008 \pm 0.002$ ,  $t = 3.497$ ,  $p = 0.007$ ; oyster:  $R^2 = 0.257$ , estimate =  $0.014 \pm 0.007$ ,  $t = 2.111$ ,  $p = 0.064$ ).

(0.542), indicating that *M. orientalis* pays a cost of generalism (Figure 5). As expected from the absence of *M. intestinalis* infections in oysters in the field survey, we did not recover any successful infection of *M. intestinalis* in oyster hosts and can thus provide experimental confirmation that *M. intestinalis* is a specialist for blue mussels (Figure 5). For the host source dependence analysis, we therefore decided to drop this group from the analyses. Because we lacked the combination of *M. intestinalis* infection and oyster as a host, our full-factorial design collapsed and we decided to adopt an analysis strategy that first tested the infection treatment in all remaining mussel and oyster hosts. This confirmed our previous observations that *M. orientalis* had significantly lower infection success than *M. intestinalis* infections irrespective of their host origin (Figure 5; *M. orientalis* from mussel: estimate =  $-3.320 \pm 0.244$ ,  $z = -13.632$ ,  $p < 0.001$ , *M. orientalis* from oyster: estimate =  $-2.152 \pm 0.189$ ,  $z = -11.419$ ,  $p < 0.001$ ). These non-overlapping estimates relative to *M. intestinalis* in mussels suggested that *M. orientalis* originating from oysters had higher infection success than *M. orientalis* from mussels. To formally test that we fitted a binomial GLM only containing *M. orientalis* infections with source (mussel vs. oyster) and infection hosts (mussel vs. oyster) as parameters. This confirmed that *M. orientalis* had significantly lower infection success when it originated from mussels than *M. orientalis* originating from oysters (Table 1C). This difference was consistent between infection hosts and did not depend on the

combination of source and infection host (Table 1C), indicating that *M. orientalis* profits from reproducing in its principal oyster host, from where it can spill over offspring of relatively higher fitness into its newly acquired mussel host.

### 3.4 Both Parasites Differ in Their Aggregation Behavior

This experiment tested whether the observed aggregated distribution can be explained by *M. orientalis* preferentially infecting hosts with existing prior infections. While the primary infections showed the same trend of lower infection success of *M. orientalis*, we found significantly higher infection rates for *M. orientalis* than for *M. intestinalis* in the preference infection (Figure 6,  $F_{1,39} = 5.494$ ,  $p = 0.024$ ), which was independent of the primary infection ( $F_{1,39} = 0.347$ ,  $p = 0.561$ ) and the combination of primary and preference infection ( $F_{1,39} = 0.000$ ,  $p = 0.991$ ). The choice of host with and without primary infection, i.e. the infection preference, also differed between the two parasites irrespective of the overall infection rates. Here, *M. orientalis* significantly preferred hosts with primary infections over uninfected hosts in both con- and hetero-specific infections (Figure 6). In hetero-specific infections *M. orientalis* strongly preferred the infected host, whereas *M. intestinalis* seemed to avoid mussels with primary infections by *M. orientalis* (Figure 6). Consequently, we could detect a significant interaction effect between the parasite species used in the preference infection and



**FIGURE 4** | Infection rates of simultaneous infections and sequential infections. Violins show the range of the observed data with violin width representing the frequency distribution of values. Box and whiskers give the 50 and 95 percentile, and white dots within boxes show the median. **(A)** In simultaneous infections the only significant difference observed was the lower infection rates of *Mytilicola orientalis* (red) compared to *Mytilicola intestinalis* (blue), which was consistent between infection types (see **Table 1A**). *Post-hoc* tests between all groups showed the between-species comparisons were all significant ( $P < 0.001$ ), whereas the within-species comparisons were not ( $p > 0.99$ ). **(B)** Similarly, *M. orientalis* had significantly lower infection rates in sequential infections. Additionally, infection rates were on average lower in the second round of infection. This difference was however mainly driven by *M. intestinalis* infections, whereas there was no significant difference in *M. orientalis* infections (see **Table 1B**). Pairwise comparisons between single infections to the corresponding infection rates in hetero-specific infections showed no significant difference (all  $p$ -values  $> 0.9$ ), indicating that between parasite interactions did not alter the outcome of infections. Infection rates resulting from the same species in both rounds were combined into one group.

whether the infected mussel was infected with a con- or a hetero-specific parasite (**Figure 5**). We found similar overall infection rates between hetero- and conspecific infections compatible with infection preference behavior rather than competitive exclusion after infection. Such active infection choice behavior leads to high aggregation in of *M. orientalis*, but also the decline in mating opportunities for *M. intestinalis* resulting from a lack of conspecific preference and avoidance of mussels with a primary *M. orientalis* infection (**Figure 3C** and **Figure 6**).

## 4 DISCUSSION

How generalist parasites can coexist with specialists in the same niche in the face of costs to generalism remains an unsolved question (Visher and Boots, 2020). Even more so, it is an enigma how a generalist can invade a niche that is densely occupied by an established specialist. Empirical systems to address these questions are rare, especially for non-microbial parasites. By focussing on the successful and ongoing invasion of the parasitic copepod

*Mytilicola orientalis* into the niche of its specialist congener *Mytilicola intestinalis*, we could now identify mechanisms of establishment in the densely occupied intestine of blue mussels *Mytilus edulis*. Both parasites are morphologically and ecologically very similar, making competitive interactions likely (Fukami, 2015). Nevertheless, we have shown that in the absence of direct competition, indirect effects that alter the invader's epidemiology and interfere with host choice of the established species can contribute to explaining the invasion success mirrored in the ongoing invasion observed in the field. A phase shift analysis on our epidemiological field data showed that the decreasing prevalence of *M. intestinalis* was best predicted by the preceding prevalence of *M. orientalis*, suggesting a causal implication of *M. orientalis* in the decline of *M. intestinalis* during the initial stages of its invasion. *In situ* observations of parasite interactions in the wild are rare in general (Telfer et al., 2008; Fenton et al., 2010; Hellard et al., 2015), but observations of one species displacing its established congener in a natural setting offer an excellent opportunity to study the onset of competition. Furthermore, other studies investigating competitive interactions between



**TABLE 1** | Generalized linear (GLM) and Generalized linear mixed model (GLMM) model parameters.

<b>Fixed effect</b>	<b>Estimate ± se</b>	<b>z-value</b>	<b>P</b>	<b>Random effects</b>	<b>Variance</b>
<b>A) Simultaneous infections (binomial GLMM)</b>					
<b>Parasite species</b>	<b>-1.960 ± 0.300</b>	<b>-6.529</b>	<b>&lt; 0.001</b>	Mussel Id	0.851
<b>Mori vs. Mint</b>					
Infection	-0.116 ± 0.420	-0.275	0.783		
<i>Single vs. co-infection</i>					
Parasite x infection	0.072 ± 0.535	0.134	0.893		
<b>B) Sequential infections (binomial GLMM)</b>					
<b>Parasite species</b>	<b>-1.238 ± 0.454</b>	<b>-2.726</b>	<b>0.006</b>	Mussel Id	0.777
<b>Mori vs. Mint</b>					
<b>Round</b>	<b>-1.610 ± 0.462</b>	<b>-3.482</b>	<b>&lt; 0.001</b>		
<b>Round2 vs. Round1</b>					
Infection	-0.095 ± 0.474	-0.200	0.841		
<i>Homolog vs. Single</i>					
Infection	0.141 ± 0.479	0.295	0.768		
<i>Heterolog vs. Single</i>					
Parasite x Round	0.790 ± 0.654	1.209	0.227		
<i>Mori-Round2 vs. Mint-Round1</i>					
Parasite x infection	-1.021 ± 0.697	-1.466	0.143		
<i>Mori-homolog vs. Mint-Single</i>					
Parasite x infection	-1.178 ± 0.691	-1.705	0.088		
<i>Mori-heterolog vs. Mint-Single</i>					
Round x Infection	-0.874 ± 0.576	-1.517	0.129		
<i>Round2-homolog vs. Round1-Single</i>					
Round x Infection	0.100 ± 0.682	0.147	0.883		
<i>Round2-heterolog vs. Round1-Single</i>					
<b>Parasite x Round x Infection</b>	<b>2.579 ± 0.823</b>	<b>3.134</b>	<b>0.002</b>		
<b>Mori-Round2-Homolog vs. Mint-Round1-Single</b>					
Parasite x Round x Infection	1.176 ± 1.125	1.046	0.296		
<i>Mori-Round2-Heterolog vs. Mint-Round1-Single</i>					
<b>C) Host Source (binomial GLM, only using <i>M. orientalis</i> infections)</b>					
<b>Source host</b>	<b>-1.284 ± 0.359</b>	<b>-3.582</b>	<b>&lt; 0.001</b>		
<b>Mussel vs. Oyster</b>					
Infection host	-0.142 ± 0.259	-0.546	0.585		
<i>Mussel vs. Oyster</i>					
Source x Infection host	0.222 ± 0.483	0.460	0.646		

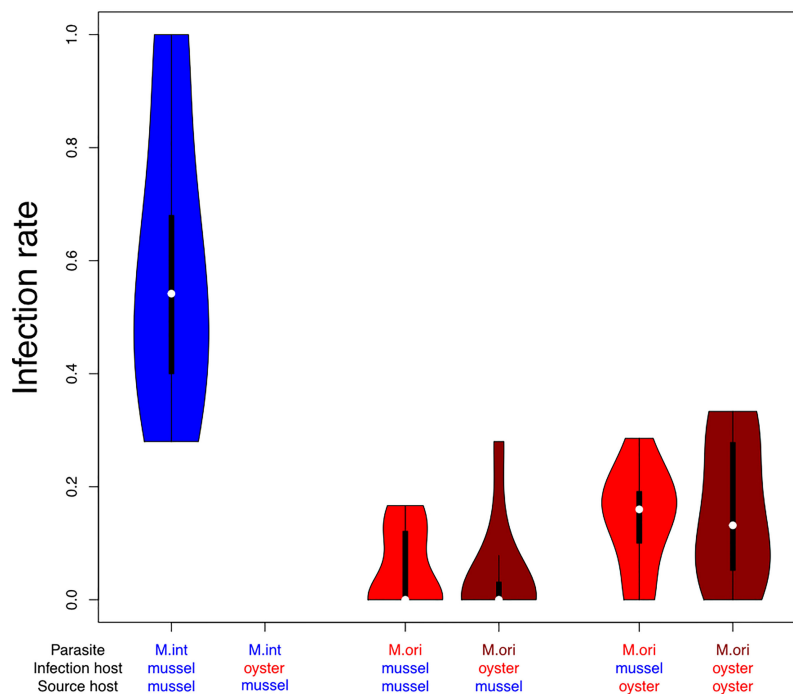
Significant fixed effects are shown in bold.

parasites use systems where interactions have evolved over evolutionary time (Lello et al., 2004) - sometimes even across speciation events (Dawson et al., 2000; Johnson et al., 2009). In the *Mytilicola* system used here, a biological invasion created novel competitors, and thus allowed us to catch a glimpse of the competitive landscapes during initial phases of such interactions.

#### 4.1 Direct Competition Cannot Explain the Success of the Newcomer

Over all infection experiments the mussel specialist *M. intestinalis* was more successful at infecting its only host than *M. orientalis* at infecting both of its hosts. Such differences in infection success could be caused by closed encounter or compatibility filters, and observational field data can in many cases not distinguish between encounter and compatibility filters as long as one of the filters remains closed (Kuris et al., 2007). With our experimental design we could however ensure that we had an open encounter filter. Therefore, differential infection success between both parasite species must rely on different compatibilities or competition between both species. The infections testing direct competitive interactions during the infection process revealed no competitive interaction between both species (i.e. single-species infections showed the same infection rates than co-infections). We had to

use allopatric sources of *M. orientalis* for the simultaneous and sequential infection experiments, which were carried out before *M. orientalis* invaded locally. Our first experiment using the identical source population for both parasite species (i.e. the host source experiment) as well as the primary infections in the preference experiment did however confirm these results with sympatric *M. orientalis*, suggesting differences between species rather than populations. Infection rates of *M. orientalis* were only higher than those of *M. intestinalis* in the preference infection of our last infection choice experiment. This difference partly arose from comparatively low infection rates of *M. intestinalis*. Together with the difficulty of finding gravid *M. intestinalis* females in 2020 (Theising & Wegner, personal observation), variation in infection rates might suggest a decline in population mean fitness of *M. intestinalis*. This effect could further contribute to the low abundance observed in the field. On the other hand, *M. intestinalis* was shown to adapt quickly to its local host population along separate evolutionary trajectories (Feis et al., 2016; Feis et al., 2018), indicating that there is substantial evolutionary potential, which might also be found in *M. orientalis*. Although co-infection can select for enhanced competitive abilities between parasite species (de Roode et al., 2005), it seems unlikely that *M. orientalis* shifted the infectiveness



**FIGURE 5** | Infection rates of *Mytilicola intestinalis* (*M. int.*, blue) and *Mytilicola orientalis* (*M. ori.*, red, dark red) originating from mussel or oyster source hosts infecting mussels or oysters as infection hosts. Violins show the range of the observed data with violin width representing the frequency distribution of values. Box and whiskers give the 50 and 95 percentile, and white dots within boxes show the median. Infection rates of *M. intestinalis* were highest in mussels, whereas none of the experimental *M. intestinalis* infections were successful in oysters. *Mytilicola orientalis* had lower infection rates in both hosts combined than *M. intestinalis* in its only host. When originating from oysters *M. orientalis* showed a higher infectivity in both hosts, but they were still less successful than *M. intestinalis* in infecting mussels.

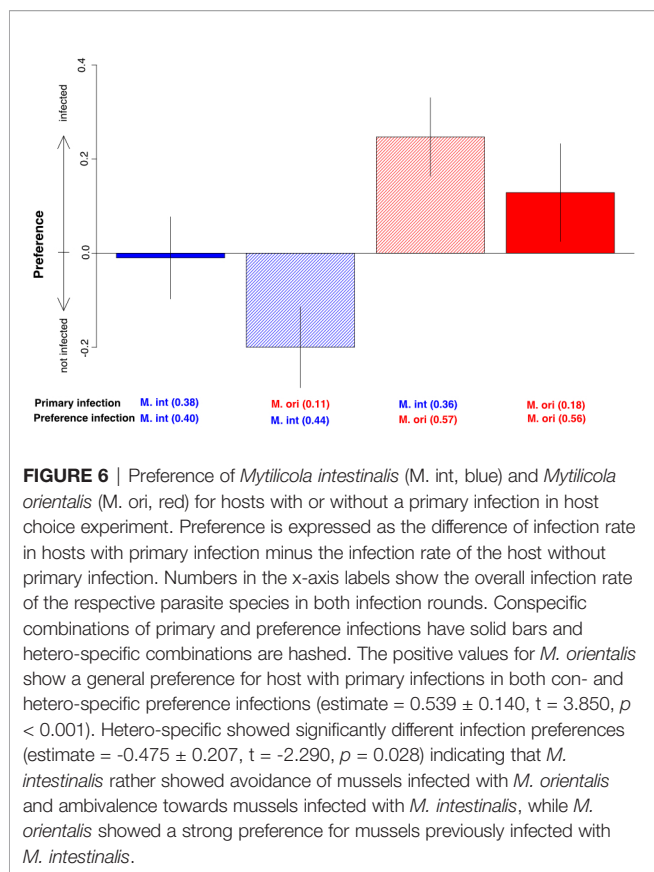
scale over a time period of a few generations. The low infection success of *M. intestinalis* in the second round of the sequential infection experiment and the high infection success of *M. orientalis* in the preference infection rather suggest that variation in infection success can be high in both species, and further experiments are needed to track the ongoing evolution of infectivity and competitive abilities in this system.

Overall, our results suggest that the decline of *M. intestinalis* did not occur because of direct competition between the two parasites in their shared mussel host. In fact, the only indication for some interference between both parasites was the lower infection rate of *M. orientalis* followed by *M. intestinalis* in sequential infections (**Figure 3B**). While the observed difference was not statistically significant, it may nevertheless indicate that prior *M. orientalis* infections might get aborted at a higher rate when followed by *M. intestinalis* infections. This would put *M. orientalis* at a competitive disadvantage, since any kind of competition is predicted to prevent the establishment of rare parasites (Quigley et al., 2018). Other studies also found such antagonistic priority effects in sequential infections (Hoverman et al., 2013), but also all other outcomes ranging from no interaction (Zilio and Koella, 2020) to superiority in simultaneous infections (Okabe et al., 2012) or facilitative interaction in sequential infections (Halliday et al., 2020) have

been observed. The variety of these outcomes suggests that with extended contact over evolutionary time different co-evolutionary trajectories can be observed depending on the specific ecological conditions encountered during contact. In our case, the absence of direct competition between both parasite species surely helped the initial establishment of *M. orientalis* in its new mussel host.

## 4.2 Indirect Effects on Epidemiology and Host Choice Amplify Each Other

To fully explain *M. orientalis*' invasion success into the occupied niche, alternative mechanisms are needed. In direct comparison *M. orientalis* already displayed higher resilience to changing environmental conditions (Brenner et al., 2019) indicating that this species has also evolved higher tolerance to abiotic conditions. However, tolerance to external environmental conditions mainly affects the free-living transmission stage, while within host environments can be considered as habitats that are buffered from abiotic fluctuations. Therefore, we focused our investigations on host-related factors and our first hypothesis was that the origin from the alternative hosts of *M. orientalis* parasite produces offspring of different quality. We could confirm this hypothesis, since infections originating from egg sacs collected from oyster hosts showed higher infection success than offspring from mussel hosts. This suggests that maturation and reproduction



in the principal oyster host generates offspring of higher fitness that feeds back positively on the epidemiology in the mussel host. If a high proportion of mussel infections originate from oyster hosts, continuous spillover will provide a surplus in infective propagules able to infect the newly acquired host (Goedknecht et al., 2016b), thereby increasing overall transmission success. Indeed, only in the very first stages of the invasion *M. orientalis* prevalences were higher in oysters than in mussels (Figure 1B). Infection intensities, on the other hand, always remained higher in oysters, suggesting that an increasing number of mussels got infected from oyster reservoir hosts where high infection intensities produced a large number of infective offspring with higher transmission probability (Figure 5). Furthermore, dilution effects, i.e. the loss of infective stages by ending up in the wrong host species (Thieltges et al., 2008), are asymmetric between both *Mytilicola* species and their hosts (Goedknecht et al., 2019). The lower transmissibility of *M. orientalis* originating from mussels can be considered as a minor dilution for *M. orientalis* epidemiology. However, the incompatibility of *M. intestinalis* and oyster hosts indicates that *M. intestinalis* can lose many infective stages in oysters due to a dilution effect caused by a closed compatibility filter (Kuris et al., 2007). Dilution of *M. intestinalis* is further amplified by the modification of host preference depending on prior infections. *Mytilicola intestinalis* copepodites avoided mussels infected with *M. orientalis*, while *M. orientalis* copepodites preferentially infect mussels with prior infections (especially infection with *M. intestinalis*, Figure 6). This non-competitive priority effect

generates a positive feedback where mussels with prior *M. intestinalis* infections are over-proportionally infected by *M. orientalis* originating from oyster reservoir hosts. Reservoir hosts have been shown to have strong effects on parasite epidemiology alone (Al-Shorbaji et al., 2016), and the growing numbers of feral oysters in the Waddensea (Reise et al., 2017a) will most certainly also lead to more *M. orientalis* infections in mussels. *M. orientalis* infections in mussels can then prevent *M. intestinalis* transmission potentially reducing *M. intestinalis* population growth. This behavior was also reflected in the field data where the overall infection prevalence was only additive when *M. intestinalis* was comparatively rare (i.e. late 2018 in Figure 1B). The combination of transmission stages with higher infectivity coming from oysters and the interference of *M. intestinalis* host finding by *M. orientalis*, makes oysters a diluter of *M. intestinalis* epidemiology and an amplifier of *M. orientalis* epidemiology at the same time. This modification of epidemiology is not accessible to *M. intestinalis* and can therefore largely contribute to invasion success of *M. orientalis*. Interestingly, the overall prevalence of both parasites combined in mussel hosts also dropped since *M. orientalis* invaded (Figure 1B). Since infection by both parasites leads to similar energetic costs and lowered body condition of the mussel host (Feis et al., 2016; Goedknecht et al., 2018a), mussels might have actually profited from the invasion of *M. orientalis* due to lower infection rates.

The population decline of *M. intestinalis* can then be further accelerated by Allee effects caused by lack of mating opportunities (Regoes et al., 2002; Berec et al., 2007). Aggregation in sexual parasites can be directly translated into potential mating opportunity, and low infection intensities will create Allee effects due to the lack of a suitable partner of opposite sex. In the context of mate limitation, the ability to detect and preferentially infect hosts with pre-existing infections can be adaptive and the observed aggregation patterns might suggest that *M. orientalis* can actively aggregate in hosts, whereas *M. intestinalis* suffered in terms of reproductive success due to the lack of aggregation ability. Even though the realized reproductive success can vary widely around the probability of encountering the opposite sex, this number sets a lower boundary that needs to be overcome. The active aggregation by preferential infection of hosts with prior infections observed for *M. orientalis* can circumvent such Allee effects, and is also needed for this species given its low prevalence in its principle oyster host. *Mytilicola intestinalis*, on the other hand, has experienced high prevalences (>90% during the reproductive season) making active choice for already infected hosts obsolete. With the onset of declining prevalences, mating opportunities then got increasingly rare, especially with the avoidance of hosts already infected with *M. orientalis*. Together, Allee effects will be amplified, and could thus further explain the accelerating decline of *M. intestinalis*.

### 4.3 Conclusion

The driving force behind the advance of *M. orientalis* at the detriment of *M. intestinalis* is not the direct competition between the species. Rather, recurrent spill-over (Goedknecht et al., 2016b) of high fitness propagules created positive effects on *M. orientalis* epidemiology, and the modification of hosts choice behavior can

further accelerate the decline of *M. intestinalis*. Both aspects can act synergistically to open the gate for dominance of *M. orientalis* over *M. intestinalis*. These mechanisms can be considered as indirect effects that help a newly introduced parasite to overcome its low compatibility and take over the niche of the established specialist without direct competition. Indirect effects in parasite interactions have so far mainly focused on host immunity (Graham, 2008; Dunn et al., 2012; Demann and Wegner, 2019), but our data shows that other mechanisms can mediate indirect interactions outside of host immunity. Such mechanistically hidden indirect parasite interactions need to be addressed in a wider array of species to understand niche breadth evolution and invasion success.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the github repository: <https://github.com/mathiaswegner-AWI/OccupiedNicheInvasion.git>.

## AUTHOR CONTRIBUTIONS

Field data was collected by MF, LG, LR, FD, and KW, and analyses by KW. MF and KW designed and carried out the

concurrent infection experiment. LG and KW designed and carried out the sequential experiment. LR and KW designed and carried out the parental effects experiment. FT and KW designed and carried out the aggregation experiment. MF, LG, LR, FT, and KW analyses the data from the experiments. MF and KW wrote the original draft of the manuscript, and all authors helped with editing. All authors contributed to the article and approved the submitted version.

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