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TRAIT-SPECIFIC TRADE-OFFS PREVENT NICHE EXPANSION IN TWO PARASITES

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ABSTRACT

The evolution of host specialization has been studied intensively, yet it is still often difficult to determine why parasites do not evolve broader niches – in particular when the available hosts are closely related and ecologically similar. Here, we used an experimental evolution approach to study the evolution of parasite specialization, and its underlying traits, in two sympatric parasites: *Anostracospora rigaudi* and *Enterocytospora artemiae*, microsporidians infecting the brine shrimp *Artemia franciscana* and *Artemia parthenogenetica*. In the field, both parasites regularly infect both hosts, yet experimental work has revealed that they are each semi-specialized. We serially passaged the parasites on one, the other, or an alternation of the two hosts; after ten passages, we assayed the infectivity, virulence, and spore production rate of the evolved lines. In accordance with previous studies, *A. rigaudi* maintained a higher fitness on *A. parthenogenetica*, and *E. artemiae* on *A. franciscana*, in all treatments. Further, we showed that the origin of this specialization was not infectivity, which readily evolved and showed a weak trade-off among host species for both parasites. Instead, there was a strong trade-off in spore production, suggesting that the ability to exploit the host and produce spores is the barrier preventing the evolution of generalism in this system. Intriguingly, the trait-specific trade-offs appear to be very similar for both parasites. This study highlights that the shape of between-host trade-offs can be very heterogeneous across parasite traits, so that only some traits are pivotal to specialization.

KEYWORDS

Ecological specialization, niche, host specificity, experimental evolution, parasite life history, multi-host parasites, *Artemia*, microsporidians.

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INTRODUCTION

Most parasites manifest a degree of specialization in nature, with niches that do not cover the entire community of potential hosts. This occurs even in communities of ecologically and physiologically similar host species (e.g. Antonovics et al. 2002, Hall et al. 2009, Streicker et al. 2013, Lievens et al. 2019), begging the question of why parasites do not evolve to extend their niche. Answering this question is particularly relevant when trying to predict the future evolution of a parasite, for example with regards to the emergence of new diseases (Cleaveland et al. 2001) or the impact of invasive hosts (Prenter et al. 2004, Kelly et al. 2009).

The evolution of host specialization – i.e. the evolution of parasitic niches – is generally studied through the lens of ecological specialization theory. A cornerstone of specialization theory is the assumption that adaptation to one environment trades off with adaptation to another (reviewed in e.g. Futuyma and Moreno 1988, Kassen 2002, Ravigne et al. 2009). The strength of the fitness trade-offs determines, to a large degree, whether specialist or generalist strategies evolve: strong trade-offs favor the evolution of specialists, while weak trade-offs favor the evolution of generalists. At first glance, therefore, we might view specialism in a parasite population as an indicator that there are strong fitness trade-offs between the hosts. This would imply that the parasite could never evolve to become a generalist. However, specialization is also governed by the availability and demography of the different environments (e.g. Bell and Reboud 1997, Ronce and Kirkpatrick 2001, Ravigne et al. 2009). Low encounter rates with the alternative host can maintain specialism in spite of weak trade-offs (Benmayor et al. 2009), especially if that host is an ecological sink (Holt and Gaines 1992, Holt and Hochberg 2002, Lenormand 2002, Ching et al. 2013). In this case, a demographic change in the host community could indeed prompt a shift towards generalism in the parasite. This scenario would seem especially likely among similar host species, where we might expect trade-offs to be weaker (cf. Hereford 2009). To disentangle the consequences of trade-offs from those of host availability, experimental evolution studies are necessary (cf. Kassen 2002, Fry 2003).

Many experimental evolution studies have been done on parasite specialization, yielding a variety of outcomes: evidence for fitness trade-offs (e.g. Turner and Elena 2000, Yourth and Schmid-Hempel 2006, Legros and Koella 2010), mixed support for trade-offs (e.g. Fry 1996, Agrawal 2000, Nidelet and Kaltz 2007, Magalhães et al. 2009, Bedhomme et al. 2012, Messina and Durham 2015), selection for generalism in fluctuating environments (e.g. Poullain et al. 2008, Bruns et al. 2014, Magalhães et al. 2014), and complex effects of migration and host availability (e.g. Benmayor et al. 2009, Ching et al.

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2013). However, experimental evolution studies that take the natural context of parasite populations into account are rare (Jaenike and Dombeck 1998, Fellous et al. 2014). In addition, few studies look for the traits underlying fitness trade-offs (Jaenike and Dombeck 1998, Magalhães et al. 2009, Bedhomme et al. 2012, Messina and Durham 2015). Parasite fitness is a composite of successful infection, host exploitation, and transmission success, and any of these traits may pose problems when adapting to new hosts. Low fitness in a new host may result from strong trade-offs in one or a few key traits, or from the accumulation of weak trade-offs in most traits. However, only strong trade-offs may prevent the evolution of a generalist parasite on the long term. Identifying whether such key traits occur is a largely unexplored, but crucial question to understand patterns of host specialization.

In this study, we investigated whether trade-offs in host use limit the evolution of specialization of two naturally occurring parasites, and if so, upon which traits these trade-offs act. We used the microsporidians *Anostracospora rigaudi* and *Enterocytospora artemiae* and their sympatric hosts, the brine shrimp *Artemia parthenogenetica* and *Artemia franciscana*. The two parasites are ecologically similar, can complete their life cycles on both hosts, and commonly infect both hosts in the field (Rode et al. 2013b). Nonetheless, they each show a degree of specialization: *A. rigaudi* has much higher fitness in *A. parthenogenetica* and cannot persist without this host in the field, while *E. artemiae*'s fitness is much higher in *A. franciscana* (Lievens et al. 2018, 2019). To disentangle the effects of host availability from trade-offs for these semi-specialized parasites, we manipulated their host environment by serially passaging them on one, the other, or an alternation of the two hosts. We then assayed the infectivity, virulence, and spore production rate of the evolved lines, and asked: [1] did manipulating the host environment affect the degree of specialization of the parasites?; [2] what was the role of the underlying traits?; and [3] were these results consistent with trade-offs in host use?

METHODS

Hosts and parasites

Natural system

Artemia is a genus of small crustaceans occurring in hypersaline environments. Our study system, the saltern of Aigues-Mortes on the Mediterranean coast of France, contains two sympatric *Artemia* species. The first, *A. parthenogenetica*, is an asexual clade native to the area; the second, *A. franciscana*, is a sexual species that was introduced from North America in 1970 and has since become highly prevalent

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(Amat et al. 2005, Rode et al. 2013b). The two species coexist for most of the year, and can usually be found in the same microhabitats (Lievens et al. 2019).

A. rigaudi and *E. artemiae* are two of the most prominent parasites infecting *Artemia* in Aigues-Mortes, with infections of either reaching prevalences of up to 100% in both host species (Lievens et al. 2019). Both microsporidians are horizontally transmitted parasites of the gut epithelium: once established, infections continuously release spores into the environment with the host's feces (Rode et al. 2013a). Experiments revealed that the fitness of *A. rigaudi* and *E. artemiae* is higher when infecting respectively *A. parthenogenetica* and *A. franciscana* (Lievens et al. 2019). This is mainly due to differences in spore production, although *E. artemiae* is also a poor infector of *A. parthenogenetica* and *A. rigaudi* has suboptimal virulence in *A. franciscana*.

Origin of experimental parasites

We obtained our experimental parasites from the same laboratory stocks of *A. rigaudi* and *E. artemiae* that were used by Lievens et al. (2019) to estimate infectivity, virulence, and spore production. The microsporidians in these stocks were collected in Aigues-Mortes and maintained on a mix of both hosts. Before starting the serial passages, we made sure that the stocks were singly infected by using them to infect lab-bred hosts, testing those hosts for the presence of both microsporidians, and re-starting the stocks from singly infected hosts only (see Supplementary Material for more details). Note that although we tried to maximize the genetic diversity of our stocks by using spores produced by both host species, originating in several sites and at different times, we do not know if the resulting microsporidian populations were genetically diverse or not.

Experimental evolution

We serially passaged the microsporidians *A. rigaudi* and *E. artemiae* on the host species *A. franciscana*, *A. parthenogenetica*, or an alternation of the two. After 10 passages, we assayed the infectivity, virulence, and spore production of each line, and compared these among treatments.

Experimental conditions

See Supplementary Methods.

Serial passages

We subjected A. rigaudi and E. artemiae to serial passaging under three evolutionary treatments: 'A. f. host', 'A. p. host', and 'Alternating hosts'. In the first two regimes, the parasites encountered only A.

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franciscana or only A. parthenogenetica; in the third regime, the parasites encountered alternating passages of A. franciscana and A. parthenogenetica. Each microsporidian × treatment combination was replicated four times, producing a total of 24 parasite lines. Parasites underwent ten serial passages, each lasting three weeks. The protocol is depicted in Fig. 1; details can be found in the Supplementary Methods.

Two aspects of our passaging protocol should be pointed out: first, the time between passages (three weeks) is enough to allow infections to be transmitted within passaged groups (Rode et al. 2013a). Thus, low infection rates at the start of a passage could be compensated by high within-passage transmission. Second, we did not control the number of spores that were transmitted from one group of hosts to the next. Thus, the size of the inoculum and of the microsporidian population in all passages after P1 were dependent on the infection dynamics that developed within each replicate line.

Final assays

At the end of the serial passage experiment, we tested the infectivity, virulence, and spore production of each evolved line in both *A. franciscana* and *A. parthenogenetica*. We tested all surviving parasite lines based on the spores they produced at the end of P10, and an additional subset of the parasite lines based on backup spores collected after P6 (see Results). Details on spore collection after P6 and P10 can be found in the Supplementary Methods.

We performed two final assays (described in Fig. 1, details in the Supplementary Methods). The first assay tested the infectivity of the evolved lines; it was replicated on 40 *A. parthenogenetica* and 40 *A. franciscana* individuals for each line. In parallel with the first, a second assay tested the virulence and spore production of each evolved line. This assay was also replicated on 40 *A. parthenogenetica* and 40 *A. franciscana* per evolved line. Because we suspected that there would be fewer infections when *A. parthenogenetica* was exposed to *E. artemiae*, we increased the level of replication for these combinations, adding an extra 20 *A. parthenogenetica*. We also included 80 control *A. parthenogenetica* and 80 control *A. franciscana*, which were not exposed to spores but otherwise treated identically. Spore production was measured by quantifying the number of spores produced by infected individuals over a two-day period after three weeks of infection; this corresponds to the window for transmission during the serial passage experiment.

Statistical analyses

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All analyses were carried out in R version 3.5.1 (R Core Team 2014), using the packages lme4 (linear mixed modeling, Bates et al. 2015) and survival (survival analyses, Therneau 2014). Unless stated otherwise, we built full models with the relevant experimental factors, and tested for the significance of effects using the likelihood ratio test. If post-hoc testing was necessary, we used Tukey HSD tests from the packages multcomp (Hothorn et al. 2008) and lsmeans [ref].

Serial passages

During the serial passage experiment, we collected data on host survival and parasite population size. Here, we tested whether these variables changed over the course of the experiment.

Host survival was quantified as the proportion of surviving hosts in each line at the end of each passage. Because we did not maintain "control" host populations during the serial passage experiment, host survival is relative (e.g. survival in 'Alternating hosts' vs. '*A. f.* host' treatments), and can only be compared within host species; we therefore analyzed it separately for *A. franciscana* and *A. parthenogenetica*. Linear mixed models included survival as a binomial response variable, *Treatment*, *Passage number* (as a continuous variable measuring time), *Parasite species* and all interactions as fixed effects, and *Line* as a random effect. In addition, we included *Passage* as a random factor, to control for background variation in the quality of the hosts. Lines where parasites were lost (see Results) were excluded.

To test whether the parasite population size changed, we built linear mixed models including *Treatment*, *Passage number* (as a continuous variable measuring time), and their interaction as fixed effects, and *Line* as a random variable. *A. rigaudi* and *E. artemiae* lines were analyzed separately. The population size was *ln*-transformed, and zero counts (lost lines) were excluded.

Final assays

In the final assays, we tested the effects of the passaging treatment on the infectivity, virulence, and spore production of the two parasites; we then compared a composite measure of parasite fitness. For each variable described below, analyses proceeded as follows. *A. rigaudi* and *E. artemiae* lines were analyzed separately. We began by testing whether surviving and revived lines were different, looking only at those treatments that included revived lines (models with fixed effects *Revival*, *Treatment, Assay host*, and their interactions). If they were not different, the revived lines were included in the subsequent analyses (models with fixed effects *Treatment*, *Assay host*, and their interaction). *Line* was always included as a random variable, or as a frailty variable for survival analyses.

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Infectivity was analyzed as the proportion of infected individuals at the end of the first assay (a binomial response) in a generalized linear mixed model. For virulence, the effects of *Treatment* were tested using log-logistic survival models, stratified over *Assay host* (this allowed the host species to have a different baseline survival shape). So that the results could be interpreted in terms of survival relative to uninfected hosts, we included the survival data of the control hosts as an additional *Treatment* category. However, we excluded any hosts that had been exposed to a parasite but not infected (cf. Lievens et al. 2018). We also excluded any hosts that died before day 11 of the assay, because infection could not be reliably detected before this day (see Supplementary Methods). To analyze the effects on spore production, we used the spore count in the fecal sample as a negative binomial response variable in a generalized linear mixed model. Fecal samples were only pooled for infected individuals; uninfected hosts were therefore implicitly excluded from the model.

Finally, we used spore production and infectivity to produce a composite fitness measure for each line. We used a measure of fitness that was representative for the context of the experiment, being the projected number of infections occurring if the line were passaged onto a new set of susceptible hosts. We calculated this as the total number of spores produced by the surviving individuals over a two-day period after three weeks of incubation (thus virulence is implicit), multiplied by the infectiousness of a single spore. Infectiousness, the probability of a single spore to start an infection, was calculated based on the results of the first assay. Following an independent action model with birth-death processes, the infectiousness of one spore is $-\ln\left(\frac{noninfected}{exposed}\right)/D$, where *D* is the spore dose, in our case 750 spores (Schmid-Hempel 2011, pg. 225-6). We analyzed fitness using a linear mixed model, after *ln*+1 transformation.

RESULTS

Serial passages

Of the twenty four parasite lines, four were lost during passaging (Table 1). Two of these were lost before we could collect a backup spore sample after P6; of the other two, only one line's P6 spores could be revived and used in the final assays.

At the beginning of passages P5, P6, and P7, exceptionally high mortality occurred in several groups of new hosts as they were being exposed to the parasites produced by the old hosts (Table 2). These episodes were concentrated in the treatments '*A. f.* host'. Notably, the passaging step from P5 to P6 was

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repeated three times without success for the line *E. artemiae* × '*A. f.* host' – Replicate 4. The transfer was eventually achieved after 6 weeks of incubation in the P5 hosts, as the other lines were being passaged from P6 to P7. We denote this transfer as 'P5 → P7' for consistency, but P7 is only the 6th passage for this particular line. To investigate whether these effects were due to increased virulence or demographic effects (increased parasite load), we included backup spores produced by these lines in the final assays (see below).

Host survival was not constant throughout the serial passage experiment, even when the background variation in host quality was taken into account (Fig. 2). As the passages progressed, the survival of *A. franciscana* in *A. rigaudi* × '*A. f.* host' lines decreased as compared to the others (significant triple interaction, $\chi^2(2) = 5.5$, $p = 0.02$, Supp. Table 1; post-hoc $-4.1 < z < -2.5$, $0.0001 < p < 0.06$). Because we could not separate the background host mortality from parasite-induced effects, we cannot say whether this change was due to increasing parasite-induced mortality in the *A. rigaudi* × '*A. f.* host' lines, or to decreasing parasite-induced mortality in the other lines. For *A. parthenogenetica*, survival rates became progressively higher in *A. rigaudi* relative to *E. artemiae* lines, as well as in 'Alternating hosts' relative to '*A. p.* host' lines (significant effects of *Parasite species* and *Treatment* in interaction with *Passage number*, $\chi^2(1) = 11.4$ and 8.6, $p < 0.001$ and $p < 0.01$, respectively, Supp. Table 1). Again, we could not distinguish between positive changes in *A. rigaudi* and 'Alternating hosts' lines or negative changes in *E. artemiae* and '*A. p.* host' lines.

The estimated population size of the parasites also varied through time (Fig. 3). For *A. rigaudi*, the population grew over the course of the experiment ($\chi^2(1) = 10.0$, $p < 0.01$ for *Passage number*, Supp. Table 1), and was significantly larger for lines evolving on *A. parthenogenetica* than for lines evolving on *A. franciscana* ($\chi^2(2) = 7.2$, $p = 0.03$ for *Treatment*, Supp. Table 1; post-hoc $z = 2.7$, $p = 0.02$). For *E. artemiae*, only the passaging regime impacted the population size, which was significantly higher in lines evolving on *A. franciscana* than in those evolving on *A. parthenogenetica* ($\chi^2(2) = 10.4$, $p < 0.01$ for *Treatment*, Supp. Table 1; post-hoc $z = 3.3$, $p < 0.01$).

Final assays

During the final assays, we tested all surviving evolved lines, as well as a set of lines revived from the backup P6 spore samples (Table 3). These included all the lines in the parasite/treatment combination *E. artemiae* × '*A. f.* host', most of which experienced a period of exceptional mortality during the transmission events before the end of P6 (Table 2). The two *E. artemiae* × 'Alternating hosts' lines whose P6 hosts were *A. franciscana* (Replicates 1 & 2) were also revived to act as controls for the effect of

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storage, but revival was only successful for Replicate 2. Finally, we succeeded in reviving the spores of the lost line *A. rigaudi* × 'A. f. host' – Replicate 2.

In the first assay, we tested for effects of passaging treatment on infectivity (Fig. 4, replicates shown in Supp. Fig. 1). The infectivity of *A. rigaudi* was unaffected by storage effects ($\chi^2(1) = 1.6$, $p = 0.21$), and did not change in response to passaging treatment ($\chi^2(2) = 2.8$, $p = 0.25$, Supp. Table 2); it tended to be higher in *A. parthenogenetica* ($\chi^2(1) = 3.7$, $p = 0.054$, Supp. Table 2). In contrast, the infectivity of *E. artemiae* was reduced by storage at 4°C ($\chi^2(1) = 4.6$, $p = 0.03$; dashed lines in Supp. Fig. 1), so the revived lines were excluded from further analysis. The infectivity of surviving *E. artemiae* lines was generally higher in *A. franciscana* than in *A. parthenogenetica*, but the difference was less strong after passaging on 'Alternating hosts' and '*A. p.* host' ($\chi^2(2) = 8.1$, $p = 0.02$ for interaction effect, Supp. Table 2).

In the second assay, we tested for effects of passaging treatment on virulence and spore production. As expected, we detected infection in the majority of the exposed hosts in all host-parasite combinations except A. parthenogenetica-E. artemiae (Table 4). No infection was detected for the line *A. rigaudi* × '*A. p.* host' – Replicate 1, so it was excluded from further analyses.

For both *A. rigaudi* and *E. artemiae*, parasite-induced mortality was unaffected by storage at 4°C (respectively $\chi^2(1.0) = 0.2$, $p = 0.64$ and $\chi^2(1.2) = 0.9$, $p = 0.39$). The revived lines were therefore included in the analyses. Overall, mortality was higher for *A. parthenogenetica*. For *A. rigaudi*, there was an additional effect of passaging treatment: when assayed on *A. parthenogenetica*, virulence was highest for lines passaged on *A. franciscana*, intermediate for lines passaged on alternating hosts, and lowest for lines passaged on *A. parthenogenetica* itself ($\chi^2(3.0) = 10.4$, $p = 0.02$ for interaction effect, Supp. Table 2). For *E. artemiae* lines, background mortality was also higher for *A. parthenogenetica* ($\chi^2(4) = 58.1$, $p < 0.0001$, Supp. Table 2), but infected hosts did not die faster than controls: virulence was not affected by passaging treatment, nor by the interaction between treatment and assay host ($\chi^2(3.6) = 3.7$, $p = 0.38$ and $\chi^2(4.5) = 5.8$, $p = 0.27$, respectively, Supp. Table 2; Fig. 4, replicates shown in Supp. Fig. 2).

Similarly, spore production at passaging was unaffected by storage at 4°C ($\chi^2(1) = 0.2$, $p = 0.69$ for *A. rigaudi*; $\chi^2(1) = 0.8$, $p = 0.38$ for *E. artemiae*), so all lines were included in the further analyses. Spore production was higher in *A. parthenogenetica* for *A. rigaudi* and in *A. franciscana* for *E. artemiae* ($\chi^2(1) = 14.8$ and $= 16.5$, $p = 0.0001$ and < 0.0001, respectively, Supp. Table 2). However, there were no effects of treatment, nor of the interaction between treatment and assay host ($\chi^2(2) \leq 0.7$ and ≤ 1.7, $p \geq 0.71$ and ≥ 0.43, respectively, Supp. Table 2; Fig. 4, replicates shown in Supp. Fig. 3).

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Finally, we analyzed an overall fitness measure for each line: the projected number of hosts that would be infected at passaging (Fig. 5). For *A. rigaudi*, storage at 4°C had no effect on the composite traits of fitness (see above), so the single revived line was included in the analysis. We also included the line *A. rigaudi* × '*A. p.* host' – Replicate 1, which failed to infect hosts in the second assay, with fitness set to 0 (excluding the line did not change the results). *A. rigaudi* fitness was always higher when tested on *A. parthenogenetica*, with no effect of passaging treatment, or of the interaction between treatment and assay host ($\chi^2(1) = 11.9$, $\chi^2(2) = 2.2$ and 2.5, $p < 0.001$, = 0.34 and = 0.29, respectively, Supp. Table 2). For *E. artemiae*, in contrast, the patterns of fitness mirrored those of infectivity. As storage at 4°C affected infectivity (see above), the revived lines were excluded. *E. artemiae* fitness was always lower in *A. parthenogenetica*, but less so after passaging on 'Alternating hosts' and '*A. p.* host' ($\chi^2(2) = 6.4$, $p = 0.04$ for interaction effect, Supp. Table 2).

DISCUSSION

We investigated the evolution of host specialization, and its underlying traits, in the microsporidian parasites *A. rigaudi* and *E. artemiae*. In the field, these parasites infect two sympatric species of *Artemia*, each with a degree of host specialization: *A. rigaudi* is preferentially adapted to *A. parthenogenetica*, and *E. artemiae* to *A. franciscana* (the "matched" hosts, Lievens et al. 2018). To test whether this pattern is shaped by host availability or by fitness trade-offs, we experimentally evolved the parasites on one or both of their natural hosts. We found that the parasites remained semi-specialized in all passaging conditions. The different parasite traits did not play an equal role in this outcome: spore production remained specialized in both parasites, infectivity readily evolved towards generalism, and virulence played a minor role. Our results are consistent with a strong trade-off acting on spore production and a weak trade-off on infectivity, and suggest that spore production is the key trait preventing the evolution of generalism in this system.

The evolution of specialization and its underlying traits

Our first conclusion is that both *A. rigaudi* and *E. artemiae* display a robust pattern of specialization: the fitness of both microsporidians was higher in the matched hosts than in the mismatched hosts, even after extended passaging on the latter (Fig. 5). This result is consistent with our previous ecology- and life history-based findings (Lievens et al. 2018, 2019), and aligns with the general host-conservative behavior of microsporidians (Smith 2009).

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295 *A. rigaudi*'s specialization for *A. parthenogenetica* was caused by a disparity in spore production. This
296 parasite produced many more spores in *A. parthenogenetica*. Neither infectivity nor spore production
297 changed detectably during the serial passages, but the passaging treatment did affect virulence (Fig. 4).
298 When tested in *A. parthenogenetica*, *A. rigaudi* lines that had evolved on that host were less virulent
299 than lines that had evolved on *A. franciscana*. Whether this was due to an incidentally high virulence on
300 a 'novel' host, or to an adaptive decrease in virulence on a 'known' host, is unknown, but both are
301 plausible (Woolhouse et al. 2001, Alizon et al. 2009). The effect of virulence on overall fitness was minor,
302 however, so the parasite stayed equally specialized for *A. parthenogenetica* in all treatments (Fig. 5).
303 These results correspond well with previous findings (Lievens et al. 2018).

304 For *E. artemiae*, specialization was apparent for spore production and infectivity. *E. artemiae* spores had
305 a higher chance of infecting *A. franciscana*, and *E. artemiae* infections also produced more spores in *A.*
306 *franciscana* (Fig. 4). Compounded, these two traits produce a clear pattern of specialization (Fig. 5).
307 Unlike that of *A. rigaudi*, however, *E. artemiae*'s fitness did evolve in some treatments. *E. artemiae* lines
308 whose passaging history included *A. parthenogenetica* had a higher fitness on this host, while their
309 fitness in *A. franciscana* was not detectably changed (compare cross & circle to triangle in Fig. 5). *E.*
310 *artemiae* can thus evolve a more generalist strategy without a detectable trade-off. This observation
311 supports the mounting evidence that "costs" of adaptation to different environments may not always be
312 present, as expected theoretically (Lenormand et al. 2018) and observed empirically (Falconer 1990,
313 Agrawal 2000, Kassen 2002, Nidelet and Kaltz 2007, Magalhães et al. 2009, Bedhomme et al. 2012,
314 Remold 2012, Gallet et al. 2014, Messina and Durham 2015). *E. artemiae*'s fitness change was driven by
315 a change in infectivity, while virulence and spore production were static. Interestingly, changes in
316 infectivity have also been found to drive the evolution of specialists and generalists in the
317 microsporidian *Brachiola algerae*, although in this case there was a correlated loss of infectivity in other
318 hosts (Legros and Koella 2010).

319 The difference in infectivity among the evolved lines of *E. artemiae* can be interpreted in two ways: its
320 infectivity in *A. parthenogenetica* either decreased when the parasite was no longer exposed to this
321 host, or increased when the parasite was forced to persist in it. We consider the second to be more
322 likely. The first possibility could be achieved by the accumulation of conditionally deleterious alleles
323 (neutral in *A. franciscana*, deleterious in *A. parthenogenetica*, Kawecki 1994) or through a loss of genetic
324 diversity (Yourth and Schmid-Hempel 2006). However, given the size of the initial inoculum and the
325 limited timeframe of the experiment, we doubt that such processes occurred. It would be easier for *E.*

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artemiae to accumulate beneficial mutations when passaged on *A. parthenogenetica*. This hypothesis is also supported by previous experimental results, which describe the infectivity of the stock population of *E. artemiae* as resembling that of the '*A. f.* host' evolved lines (Lievens et al. 2018). If so, adaptation likely occurred through an accumulation of mutations during passaging on *A. parthenogenetica*, which are beneficial in that host and neutral in *A. franciscana*. Another possibility is that beneficial mutations occurred in all passaging treatments, but that adaptation to *A. parthenogenetica* had an incidental positive effect in *A. franciscana* that matched the adaptation to the '*A. f.* host' treatment.

E. artemiae's virulence did not differ among treatments at the end of the serial passaging, and we found no evidence that it evolved over the course of the experiment. In particular, we found no evidence that the high death rates caused by *E. artemiae* in *A. franciscana* between P4 and P6 were caused by a higher virulence (Fig. 4). Instead, demographic effects were the likely culprit: *A. franciscana* does not limit *E. artemiae*'s peak spore production to a certain maximum (Lievens et al. 2018), so a higher infective dose in this combination might lead to a higher peak transmission rate, which would increase the infective dose and so on, until the number of invading spores was so high that recipient hosts were overwhelmed (e.g. Ebert et al. 2000).

Trade-offs in infectivity and spore production

An important advantage of this study is that *A. rigaudi* and *E. artemiae* share the same context: the two parasites are ecologically similar, sympatric, and infect the same host species, so we can reasonably expect that they are subject to similar life history and environmental constraints. Below, we take advantage of this to compare the evolved changes in infectivity and spore production for the two microsporidians, arriving at the compelling conclusion that the strength of their life history trade-offs is trait-dependent, but that the traits respond similarly in the two species.

The observed changes in infectivity can be explained by the existence of a weak trade-off between infectivity in *A. franciscana* and *A. parthenogenetica*. Consider first *E. artemiae*, whose ability to infect *A. parthenogenetica* improved when passaged on that host, without attendant losses in *A. franciscana*. Such cost-free adaptation could arise if the ancestral '*A. franciscana*-adapted' infectivity of *E. artemiae* was located slightly below the boundary of a weak trade-off curve (such as would be expected if the ancestral population was not perfectly adapted to the conditions of the experiment, Fry 2003). There would then be little improvement possible in *E. artemiae*'s fitness on *A. franciscana*, but a substantial improvement in *A. parthenogenetica* could easily be achieved (blue arrow in Fig. 6)(Martin and Lenormand 2015), as seen when *E. artemiae* was passaged on this host (Fig. 4). The infectivity of *A.*

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357 *rigaudi* can be interpreted in the same context. *A. rigaudi*'s ancestral infectivity is largely generalist
358 (Lievens et al. 2018), so that the potential improvements in fitness would be small in either direction,
359 thus producing the unchanged infectivity that we observed across treatments (Fig. 4).

360 The weak trade-off model implies that the evolution of generalist infectivity should be straightforward,
361 begging the question of why *E. artemiae*'s ancestral population remained specialized for this trait. We
362 speculate that the specialization of the ancestral *E. artemiae* population is maintained by source-sink
363 dynamics in the natural host-parasite community. In the field, *E. artemiae* is present year-round, while
364 *A. parthenogenetica* are only present from late spring to fall, so its population predominantly infects,
365 and evolves on, *A. franciscana* (Lievens et al. 2019). In this case, adaptations towards increased
366 infectivity in the mismatched host may be continually eroded by selection in the matched host (Holt and
367 Hochberg 2002, Lenormand 2002). By forcing *E. artemiae* to evolve on *A. parthenogenetica*, we blocked
368 these source-sink dynamics, allowing generalist infectivity to evolve. In comparison, *A. rigaudi* almost
369 exclusively occur in communities containing both host species (Lievens et al. 2019), potentially
370 explaining why this microsporidian had already evolved generalist infectivity.

371 The second important trait for *A. rigaudi* and *E. artemiae* was spore production, which remained
372 strongly specialized in all treatments (Fig. 4). This result could be explained by the existence of a strong
373 trade-off in spore production between the hosts. Such a trade-off would allow small improvements in
374 the direction of increased specialization (black arrows in Fig. 6), but make improvements on the novel
375 host much more difficult to achieve (red arrows in Fig. 6); it would also prevent the emergence of a
376 generalist phenotype. Mechanistically, such a strong trade-off could be related to the distinct strategies
377 of host exploitation necessary to thrive in *A. franciscana* and *A. parthenogenetica*. The precise
378 physiology of the host species is likely to be different (they have been diverging for an estimated 40
379 million years, Baxevanis et al. 2006), and indeed the mechanisms of virulence and within-host regulation
380 employed by *A. rigaudi* and *E. artemiae* in their matched hosts differ (Lievens et al. 2018, Lievens et al.
381 unpublished data), with *A. rigaudi* causing more survival virulence, and *E. artemiae* more reproductive
382 virulence. Successful exploitation of *A. franciscana* and *A. parthenogenetica* could therefore require very
383 different toolkits, preventing the evolution of generalism and reducing the likelihood of a host switch
384 (cf. Gemmill et al. 2000).

385 Taken together, our results provide strong evidence that the microsporidians' traits are constrained by
386 different trade-off shapes. Intriguingly, while the trade-offs are trait-specific, they are not species-
387 specific. It seems that while *A. franciscana* and *A. parthenogenetica* are not physiologically similar

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388 enough to allow the evolution of generalism, *A. rigaudi* and *E. artemiae* are ecologically similar enough
389 to share the same constraints.

390 *Perspectives*

391 Overall, we find that the natural semi-specialization of *A. rigaudi* and *E. artemiae* is primarily shaped by
392 a strong trade-off acting on spore production. Spore production is therefore a key trait blocking the
393 evolution of generalism. However, host availability did affect the degree of specialization, by allowing
394 the evolution of generalist infectivity. We therefore predict that the natural population of *E. artemiae*
395 may eventually evolve to become less specific, as *A. rigaudi* is, but that neither parasite is likely to
396 become a true generalist or to switch hosts.

397 It is worth noting that our conclusions would have been very different if we had not measured the
398 parasites' traits separately. Based on the overall fitness (Fig. 5), we would have concluded that *A. rigaudi*
399 was unable to adapt to its mismatched host, while *E. artemiae* was able to evolve towards generalism
400 after exposure to *A. parthenogenetica*. This would have suggested that the two parasites had
401 asymmetrical fitness trade-offs between hosts: a strong trade-off for *A. rigaudi*, and a weaker trade-off
402 for *E. artemiae*. Ignoring the individual traits, therefore, can have important consequences for the
403 interpretation of field patterns, and for the prediction of a parasite's future evolution. Our results
404 suggest that more theoretical studies of specialization should be set in a multi-trait context, with each
405 trait able to exhibit weak or strong trade-offs and evolve accordingly. Such studies would be better
406 equipped to describe the continuum between generalist and specialist strategies, and to single out the
407 traits favoring their evolution.

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TABLES

Table 1. Parasite lines that were lost during the experiment. Underlined are the hosts from which we collected a backup spore sample.

Parasite line	Lost between
A. rigaudi × '*A. f.* host' – Repl. 2	P6 & P7
A. rigaudi × '*A. f.* host' – Repl. 3	P4 & P6
A. rigaudi × 'Alternating hosts' – Repl. 4	P1 & P4
E. artemiae × '*A. p.* host' – Repl. 3	P6 & P7

Table 2. Parasite lines that had exceptionally high mortality during one or more transmission events (≤ 5 new hosts survived). To prevent the loss of these lines, we added five extra new hosts to the group at the beginning of the incubation period. If all new hosts died, the transmission step was repeated for that line. Underlined are the hosts from which we collected a backup spore sample.

Parasite line	Passage	Details
A. rigaudi × '*A. f.* host' – Repl. 1	P6 → P7	≤ 5 P7 hosts survived; 5 hosts added
A. rigaudi × '*A. f.* host' – Repl. 2	P5 → P6	≤ 5 P6 hosts survived; 5 hosts added
	P6 → P7	≤ 5 P7 hosts survived; 5 hosts added
A. rigaudi × '*A. f.* host' – Repl. 3	P6 → P7	≤ 5 P7 hosts survived; 5 hosts added
E. artemiae × '*A. f.* host' – Repl. 1	P5 → P6	≤ 5 P6 hosts survived; 5 hosts added
	P6 → P7	≤ 5 P7 hosts survived; 5 hosts added
E. artemiae × '*A. f.* host' – Repl. 2	P4 → P5	0 P5 hosts survived; transmission repeated
	P6 → P7	≤ 5 P7 hosts survived; 5 hosts added
E. artemiae × '*A. f.* host' – Repl. 4	P4 → P5	≤ 5 P5 hosts survived; 5 hosts added
	P5 → P6	0 P6 hosts survived (three repetitions); eventually skipped
	P5 → P7	≤ 5 P7 hosts survived; 5 hosts added

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551 **Table 3.** Evolved lines used for the final assays. Abbreviations: Y, yes; N, no.

Parasite species/Treatment	Replicate	Used?	Notes
A. rigaudi × '*A. f.* host'	1	Y	
	2 revived	Y	Lost, but revived from P6 backup sample.
	3	N	Lost.
	4	Y	
A. rigaudi × 'Alternating hosts'	1	Y	
	2	Y	
	3	Y	
	4	N	Lost.
A. rigaudi × '*A. p.* host'	1	Y	
	2	Y	
	3	Y	
	4	Y	
E. artemiae × '*A. f.* host'	1 + 1 revived	Y	Revived from P6 to compare virulence.
	2 + 2 revived	Y	Revived from P6 to compare virulence.
	3 + 3 revived	Y	Revived from P6 to compare virulence.
	4 + 4 revived	Y	Revived from P5 to compare virulence.
E. artemiae × 'Alternating hosts'	1	Y	Attempted revival from P6 was unsuccessful.
	2 + 2 revived	Y	Revived from P6 to compare virulence.
	3	Y	
	4	Y	
E. artemiae × '*A. p.* host'	1	Y	
	2	Y	
	3	N	Lost, attempted revival from P6 was unsuccessful.
	4	Y	

552

553 **Table 4.** Detection of infection in the second assay. Only hosts that died after day 10 are included here, to allow for

554 the delay in detection time (see Supplementary Methods).

Parasite species	Infected hosts
A. rigaudi	
A. franciscana	66 %
A. parthenogenetica	70 %
E. artemiae	
A. franciscana	68 %
A. parthenogenetica	34 %

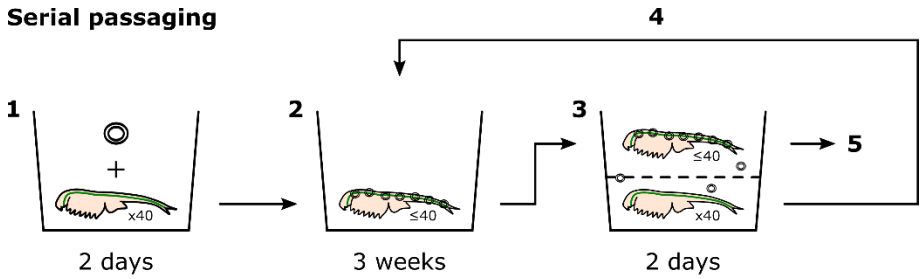
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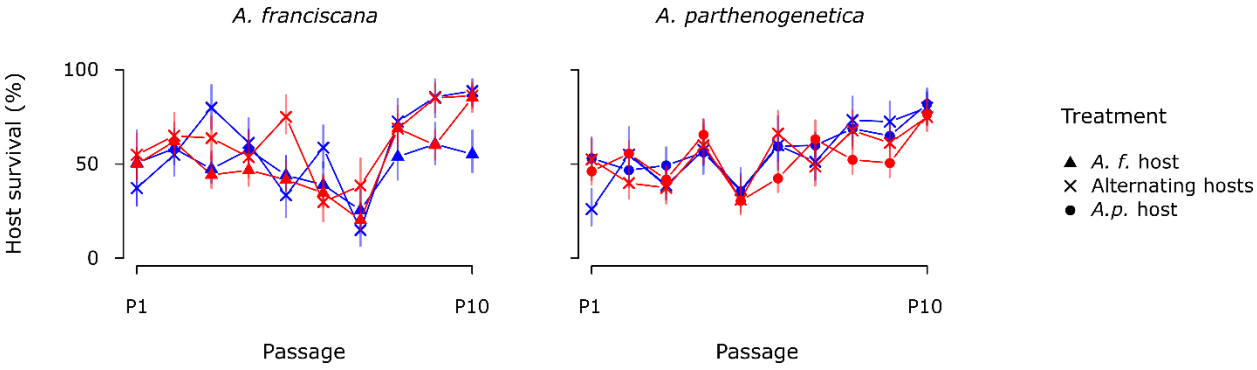
FIGURES

Figure 1. Experimental evolution protocol. **Serial passages:** [1] Passage 1 (P1): a group of 40 uninfected hosts was exposed to a saturating dose of stock *A. rigaudi* or *E. artemiae* spores, and [2] the infections were allowed to incubate. [3] Passaging (P1 → P2): the infection was transmitted naturally, by placing the surviving P1 hosts in a strainer above a new group of uninfected hosts. [4] The incubation and passaging steps were repeated for P2-P10. [5] After passaging, the surviving old hosts were counted (P1-P10) and used to estimate the population size of the parasite (P1, P4, P7), produce backup spore samples (P6), or produce the spores for the final assays (P10). **Final assays:** [1st] An uninfected host was exposed to a low dose of evolved *A. rigaudi* or *E. artemiae* spores, and PCR-tested for the presence of the microsporidian after a short incubation period. [2nd] An uninfected host was exposed to a saturating dose of evolved *A. rigaudi* or *E. artemiae* spores, and mortality and spore production were tracked for 90 days.



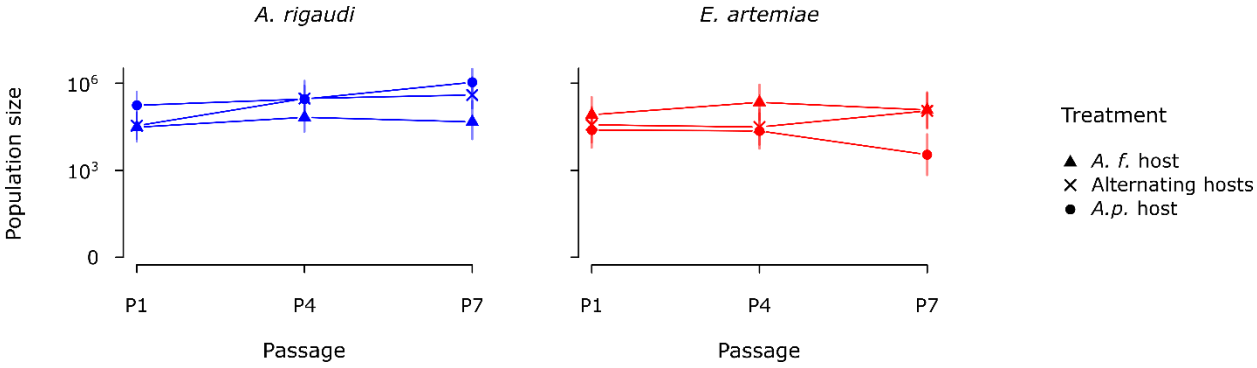
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570 **Figure 2.** Host survival during the serial passage experiment (a compound of background host mortality and
571 parasite-induced mortality). Colors denote the parasite treatment (blue, *A. rigaudi*; red, *E. artemiae*); symbols
572 denote the passaging treatment (triangles, '*A. f.* host'; crosses, 'Alternating hosts'; circles, '*A. p.* host'); vertical bars
573 represent the 95% CIs.



574

575 **Figure 3.** Parasite population size, as estimated for each passaging line (*ln* scale). Colors denote the parasite
576 treatment (blue, *A. rigaudi*; red, *E. artemiae*); symbols denote the passaging treatment (triangles, '*A. f.* host';
577 crosses, 'Alternating hosts'; circles, '*A. p.* host'); vertical bars represent the 95% CIs. Lost lines (lines with an
578 estimated population size of 0) are not included in the figure.

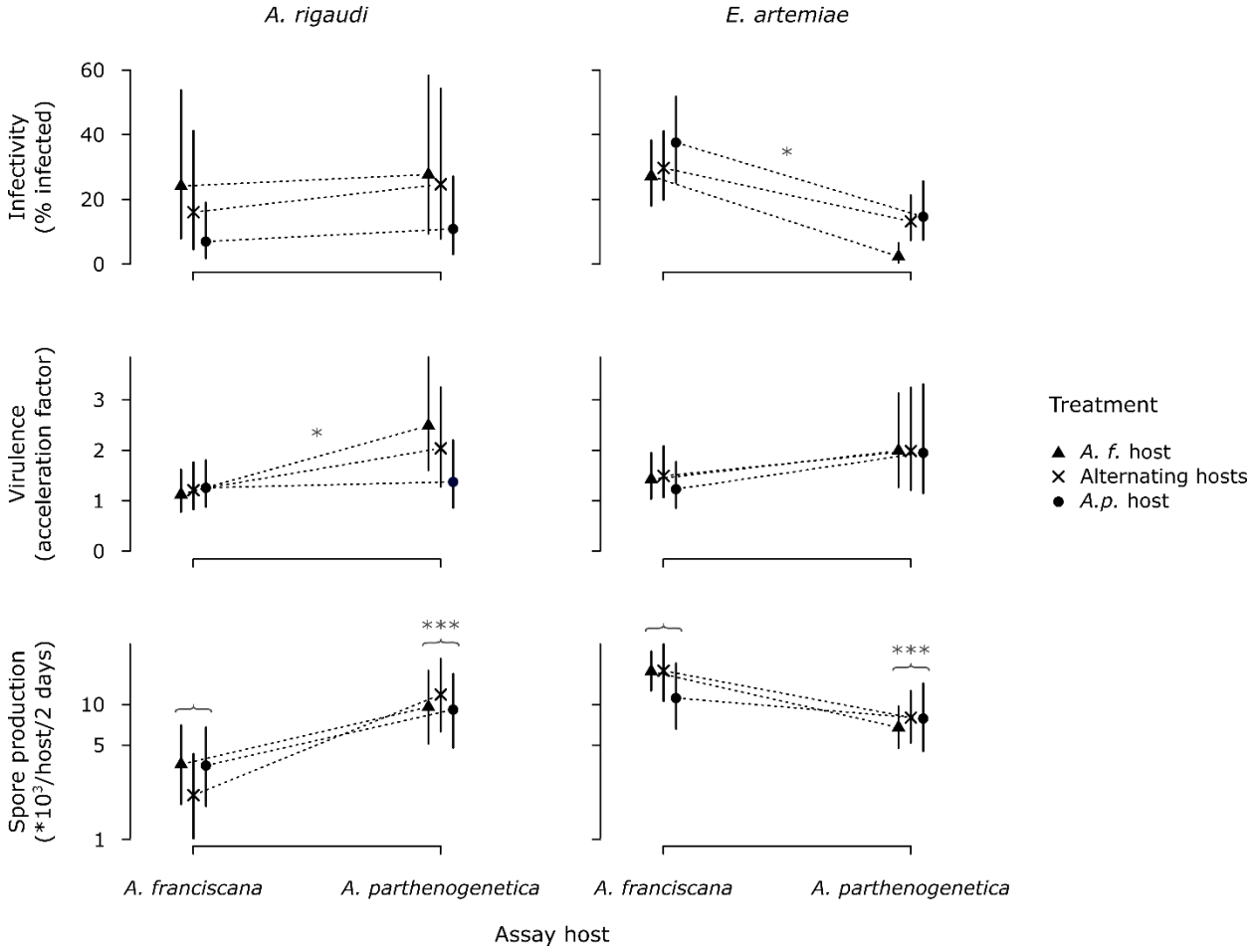


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581 **Figure 4.** Infectivity, virulence, and spore production during the final assays. Infectivity is the percentage of hosts
582 infected during the first assay. Virulence and spore production were measured in the second assay: virulence is the
583 acceleration factor (the ratio of time-until-death) compared to the unexposed controls of the same species, spore
584 production is the number of spores produced per (surviving) infected host at the time of passaging (*ln* scale).
585 Vertical bars represent the 95% CIs. Annotations in gray represent significant differences: asterisks above the
586 dashed lines indicate significant *Assay host* : *Treatment* interactions, grouped differences represent significant
587 *Assay host* differences.



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Figure 5. Fitness during the final assays (projected number of infections at passaging, *ln* + 1 scale). Vertical bars represent the 95% CIs. Annotations in gray represent significant differences: asterisks above the dashed lines indicate significant *Assay host* : *Treatment* interactions, grouped differences represent significant *Assay host* differences.

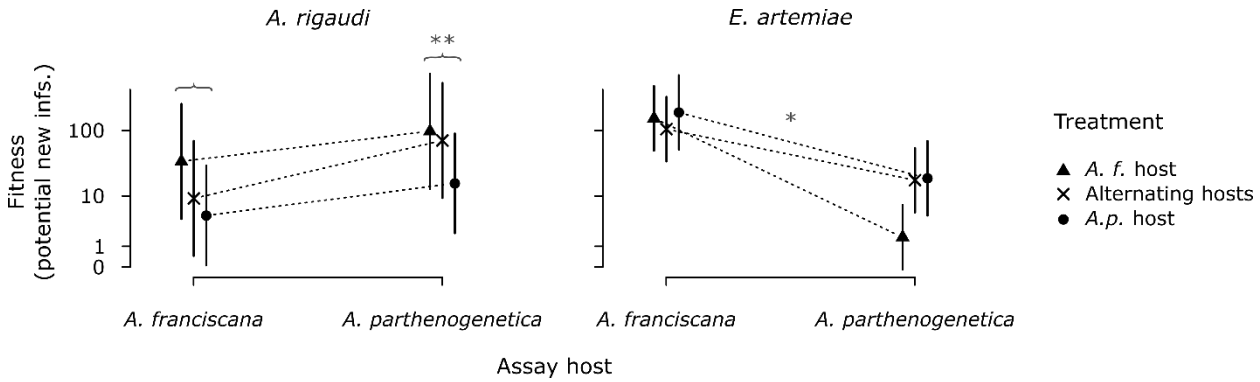


Figure 6. We speculate that the observed patterns of specialization in infectivity and spore production are determined by weak and strong trade-offs in performance between *A. franciscana* and *A. parthenogenetica* (see text for more information). Symbols: *A. rigaudi*, circle; *E. artemiae*; triangle. Black arrows, small changes are possible; blue arrow, a large change is possible; red arrows, changes in the direction of selection are not possible unless preceded by reverse specialization.

