



**HAL**  
open science

# Trait-specific trade-offs prevent niche expansion in two parasites

Eva Lievens, Yannis Michalakis, Thomas Lenormand

► **To cite this version:**

Eva Lievens, Yannis Michalakis, Thomas Lenormand. Trait-specific trade-offs prevent niche expansion in two parasites. *Journal of Evolutionary Biology*, 2020, <10.1111/jeb.13708>. <hal-02995355>

**HAL Id: hal-02995355**

**<https://hal.science/hal-02995355v1>**

Submitted on 19 Nov 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

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

2 Eva J. P. Lievens<sup>1,2</sup>, Yannis Michalakis<sup>2,+</sup> and Thomas Lenormand<sup>1,+</sup>

3 <sup>†</sup>equal contribution

4 <sup>1</sup>UMR 5175 CEFE, CNRS–Université de Montpellier–Université P. Valéry–EPHE, 1919 Route de Mende,  
5 34293 Montpellier Cedex 5, France

6 <sup>2</sup>UMR 5290 MIVEGEC, CNRS–IRD–Université de Montpellier, 911 Avenue Agropolis BP 64501, 34394  
7 Montpellier Cedex 5, France

## 8 **ABSTRACT**

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

## 25 **KEYWORDS**

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

## 28 INTRODUCTION

29 Most parasites manifest a degree of specialization in nature, with niches that do not cover the entire  
30 community of potential hosts. This occurs even in communities of ecologically and physiologically similar  
31 host species (e.g. Antonovics et al. 2002, Hall et al. 2009, Streicker et al. 2013, Lievens et al. 2019),  
32 begging the question of why parasites do not evolve to extend their niche. Answering this question is  
33 particularly relevant when trying to predict the future evolution of a parasite, for example with regards  
34 to the emergence of new diseases (Cleaveland et al. 2001) or the impact of invasive hosts (Prenter et al.  
35 2004, Kelly et al. 2009).

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

53 Many experimental evolution studies have been done on parasite specialization, yielding a variety of  
54 outcomes: evidence for fitness trade-offs (e.g. Turner and Elena 2000, Yourth and Schmid-Hempel 2006,  
55 Legros and Koella 2010), mixed support for trade-offs (e.g. Fry 1996, Agrawal 2000, Nidelet and Kaltz  
56 2007, Magalhães et al. 2009, Bedhomme et al. 2012, Messina and Durham 2015), selection for  
57 generalism in fluctuating environments (e.g. Poullain et al. 2008, Bruns et al. 2014, Magalhães et al.  
58 2014), and complex effects of migration and host availability (e.g. Benmayor et al. 2009, Ching et al.

59 2013). However, experimental evolution studies that take the natural context of parasite populations  
60 into account are rare (Jaenike and Dombeck 1998, Fellous et al. 2014). In addition, few studies look for  
61 the traits underlying fitness trade-offs (Jaenike and Dombeck 1998, Magalhães et al. 2009, Bedhomme  
62 et al. 2012, Messina and Durham 2015). Parasite fitness is a composite of successful infection, host  
63 exploitation, and transmission success, and any of these traits may pose problems when adapting to  
64 new hosts. Low fitness in a new host may result from strong trade-offs in one or a few key traits, or from  
65 the accumulation of weak trade-offs in most traits. However, only strong trade-offs may prevent the  
66 evolution of a generalist parasite on the long term. Identifying whether such key traits occur is a largely  
67 unexplored, but crucial question to understand patterns of host specialization.

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

## 81 **METHODS**

### 82 **Hosts and parasites**

#### 83 *Natural system*

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

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

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

### 98 *Origin of experimental parasites*

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

### 108 **Experimental evolution**

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

### 112 *Experimental conditions*

113 See Supplementary Methods.

### 114 *Serial passages*

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

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

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

### 128 *Final assays*

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

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

### 145 **Statistical analyses**

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

### 151 *Serial passages*

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

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

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

### 167 *Final assays*

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

176 Infectivity was analyzed as the proportion of infected individuals at the end of the first assay (a binomial  
177 response) in a generalized linear mixed model. For virulence, the effects of *Treatment* were tested using  
178 log-logistic survival models, stratified over *Assay host* (this allowed the host species to have a different  
179 baseline survival shape). So that the results could be interpreted in terms of survival relative to  
180 uninfected hosts, we included the survival data of the control hosts as an additional *Treatment* category.  
181 However, we excluded any hosts that had been exposed to a parasite but not infected (cf. Lievens et al.  
182 2018). We also excluded any hosts that died before day 11 of the assay, because infection could not be  
183 reliably detected before this day (see Supplementary Methods). To analyze the effects on spore  
184 production, we used the spore count in the fecal sample as a negative binomial response variable in a  
185 generalized linear mixed model. Fecal samples were only pooled for infected individuals; uninfected  
186 hosts were therefore implicitly excluded from the model.

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

## 197 **RESULTS**

### 198 *Serial passages*

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

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

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

211 Host survival was not constant throughout the serial passage experiment, even when the background  
212 variation in host quality was taken into account (Fig. 2). As the passages progressed, the survival of *A.*  
213 *franciscana* in *A. rigaudi* × 'A. f. host' lines decreased as compared to the others (significant triple  
214 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  
215 could not separate the background host mortality from parasite-induced effects, we cannot say whether  
216 this change was due to increasing parasite-induced mortality in the *A. rigaudi* × 'A. f. host' lines, or to  
217 decreasing parasite-induced mortality in the other lines. For *A. parthenogenetica*, survival rates became  
218 progressively higher in *A. rigaudi* relative to *E. artemiae* lines, as well as in 'Alternating hosts' relative to  
219 'A. p. host' lines (significant effects of *Parasite species* and *Treatment* in interaction with *Passage*  
220 *number*,  $\chi^2(1) = 11.4$  and  $8.6$ ,  $p < 0.001$  and  $p < 0.01$ , respectively, Supp. Table 1). Again, we could not  
221 distinguish between positive changes in *A. rigaudi* and 'Alternating hosts' lines or negative changes in *E.*  
222 *artemiae* and 'A. p. host' lines.

223 The estimated population size of the parasites also varied through time (Fig. 3). For *A. rigaudi*, the  
224 population grew over the course of the experiment ( $\chi^2(1) = 10.0$ ,  $p < 0.01$  for *Passage number*, Supp.  
225 Table 1), and was significantly larger for lines evolving on *A. parthenogenetica* than for lines evolving on  
226 *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.*  
227 *artemiae*, only the passaging regime impacted the population size, which was significantly higher in lines  
228 evolving on *A. franciscana* than in those evolving on *A. parthenogenetica* ( $\chi^2(2) = 10.4$ ,  $p < 0.01$  for  
229 *Treatment*, Supp. Table 1; post-hoc  $z = 3.3$ ,  $p < 0.01$ ).

### 230 *Final assays*

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

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

238 In the first assay, we tested for effects of passaging treatment on infectivity (Fig. 4, replicates shown in  
239 Supp. Fig. 1). The infectivity of *A. rigaudi* was unaffected by storage effects ( $\chi^2(1) = 1.6, p = 0.21$ ), and did  
240 not change in response to passaging treatment ( $\chi^2(2) = 2.8, p = 0.25$ , Supp. Table 2); it tended to be  
241 higher in *A. parthenogenetica* ( $\chi^2(1) = 3.7, p = 0.054$ , Supp. Table 2). In contrast, the infectivity of *E.*  
242 *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  
243 lines were excluded from further analysis. The infectivity of surviving *E. artemiae* lines was generally  
244 higher in *A. franciscana* than in *A. parthenogenetica*, but the difference was less strong after passaging  
245 on 'Alternating hosts' and 'A. p. host' ( $\chi^2(2) = 8.1, p = 0.02$  for interaction effect, Supp. Table 2).

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

250 For both *A. rigaudi* and *E. artemiae*, parasite-induced mortality was unaffected by storage at 4°C  
251 (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  
252 in the analyses. Overall, mortality was higher for *A. parthenogenetica*. For *A. rigaudi*, there was an  
253 additional effect of passaging treatment: when assayed on *A. parthenogenetica*, virulence was highest  
254 for lines passaged on *A. franciscana*, intermediate for lines passaged on alternating hosts, and lowest for  
255 lines passaged on *A. parthenogenetica* itself ( $\chi^2(3.0) = 10.4, p = 0.02$  for interaction effect, Supp. Table  
256 2). For *E. artemiae* lines, background mortality was also higher for *A. parthenogenetica* ( $\chi^2(4) = 58.1, p <$   
257  $0.0001$ , Supp. Table 2), but infected hosts did not die faster than controls: virulence was not affected by  
258 passaging treatment, nor by the interaction between treatment and assay host ( $\chi^2(3.6) = 3.7, p = 0.38$   
259 and  $\chi^2(4.5) = 5.8, p = 0.27$ , respectively, Supp. Table 2; Fig. 4, replicates shown in Supp. Fig. 2).

260 Similarly, spore production at passaging was unaffected by storage at 4°C ( $\chi^2(1) = 0.2, p = 0.69$  for *A.*  
261 *rigaudi*;  $\chi^2(1) = 0.8, p = 0.38$  for *E. artemiae*), so all lines were included in the further analyses. Spore  
262 production was higher in *A. parthenogenetica* for *A. rigaudi* and in *A. franciscana* for *E. artemiae* ( $\chi^2(1) =$   
263  $14.8$  and  $= 16.5, p = 0.0001$  and  $< 0.0001$ , respectively, Supp. Table 2). However, there were no effects of  
264 treatment, nor of the interaction between treatment and assay host ( $\chi^2(2) \leq 0.7$  and  $\leq 1.7, p \geq 0.71$  and  $\geq$   
265  $0.43$ , respectively, Supp. Table 2; Fig. 4, replicates shown in Supp. Fig. 3).

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

## 277 **DISCUSSION**

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

### 289 *The evolution of specialization and its underlying traits*

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

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.*

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

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

#### 341 *Trade-offs in infectivity and spore production*

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

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

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

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.

### 408 **ACKNOWLEDGEMENTS**

409 We thank C. Gilliot for her help establishing the stock populations, and C. Gilliot and T. Mathieu for their  
410 help designing the experimental equipment. We are also grateful to R. Zahab for her assistance with  
411 PCRs, and to R. Zahab, L. Olazcuaga, and J. Pantel for helping out in times of need. E. J. P. L. received  
412 financial support from a French Ministry of Research fellowship. TL and YM acknowledge support from  
413 CNRS and IRD.

414 **REFERENCES**

- 415 Agrawal, A. A. 2000. Host-range evolution: Adaptation and trade-offs in fitness of mites on alternative  
416 hosts. *Ecology* 81:500–508.
- 417 Alizon, S., A. Hurford, N. Mideo, and M. Van Baalen. 2009. Virulence evolution and the trade-off  
418 hypothesis: history, current state of affairs and the future. *Journal of Evolutionary Biology* 22:245–  
419 59.
- 420 Amat, F., F. Hontoria, O. Ruiz, A. J. Green, M. I. Sánchez, J. Figuerola, and F. Hortas. 2005. The American  
421 brine shrimp as an exotic invasive species in the western Mediterranean. *Biological Invasions* 7:37–  
422 47.
- 423 Antonovics, J., M. Hood, and J. Partain. 2002. The ecology and genetics of a host shift: *Microbotryum* as  
424 a model system. *The American Naturalist* 160:S40–S53.
- 425 Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4.  
426 *Journal of Statistical Software* 67:1–48.
- 427 Baxevanis, A. D., I. Kappas, and T. J. Abatzopoulos. 2006. Molecular phylogenetics and asexuality in the  
428 brine shrimp *Artemia*. *Molecular Phylogenetics and Evolution* 40:724–738.
- 429 Bedhomme, S., G. Lafforgue, and S. F. Elena. 2012. Multihost experimental evolution of a plant RNA  
430 virus reveals local adaptation and host-specific mutations. *Molecular Biology and Evolution*  
431 29:1481–1492.
- 432 Bell, G., and X. Reboud. 1997. Experimental evolution in *Chlamydomonas* II. Genetic variation in strongly  
433 contrasted environments. *Heredity* 78:1–11.
- 434 Benmayor, R., D. J. Hodgson, G. G. Perron, and A. Buckling. 2009. Host Mixing and Disease Emergence.  
435 *Current Biology* 19:764–767.
- 436 Bruns, E., M. L. Carson, and G. May. 2014. The jack of all trades is master of none: A pathogen’s ability to  
437 infect a greater number of host genotypes comes at a cost of delayed reproduction. *Evolution*  
438 68:2453–2466.
- 439 Ching, J., S. A. Musheyev, D. Chowdhury, J. A. Kim, Y. Choi, and J. J. Dennehy. 2013. Migration enhances  
440 adaptation in bacteriophage populations evolving in ecological sinks. *Evolution* 67:10–17.
- 441 Cleaveland, S., M. K. Laurenson, and L. H. Taylor. 2001. Diseases of humans and their domestic  
442 mammals: pathogen characteristics, host range and the risk of emergence. *Philosophical*  
443 *Transactions of the Royal Society B: Biological Sciences* 356:991–9.
- 444 Ebert, D., C. D. Zschokke-Rohringer, and H. J. Carius. 2000. Dose effects and density-dependent  
445 regulation of two microparasites of *Daphnia magna*. *Oecologia* 122:200–209.

- 446 Falconer, D. S. 1990. Selection in different environments: effects on environmental sensitivity (reaction  
447 norm) and on mean performance. *Genetical Research* 56:57.
- 448 Fellous, S., G. Angot, M. Orsucci, A. Migeon, P. Auger, I. Olivieri, and M. Navajas. 2014. Combining  
449 experimental evolution and field population assays to study the evolution of host range breadth.  
450 *Journal of Evolutionary Biology* 27:911–919.
- 451 Fry, J. D. 1996. The evolution of host specialization: are trade-offs overrated? *The American Naturalist*  
452 148:S84–S107.
- 453 Fry, J. D. 2003. Detecting ecological trade-offs using selection experiments. *Ecology* 84:1672–1678.
- 454 Futuyma, D. J., and G. Moreno. 1988. The Evolution of Ecological Specialization. *Annual Review of*  
455 *Ecology and Systematics* 19:207–233.
- 456 Gallet, R., Y. Latour, B. S. Hughes, and T. Lenormand. 2014. The dynamics of niche evolution upon abrupt  
457 environmental change. *Evolution* 68:1257–1269.
- 458 Gemmill, A. W., M. E. Viney, and A. F. Read. 2000. The evolutionary ecology of host-specificity:  
459 experimental studies with *Strongyloides ratti*. *Parasitology* 120:429–437.
- 460 Hall, S. R., C. R. Becker, J. L. Simonis, M. A. Duffy, A. J. Tessier, and C. E. Cáceres. 2009. Friendly  
461 competition: evidence for a dilution effect among competitors in a planktonic host-parasite  
462 system. *Ecology* 90:791–801.
- 463 Hereford, J. 2009. A quantitative survey of local adaptation and fitness trade-offs. *The American*  
464 *Naturalist* 173:579–88.
- 465 Holt, R. D., and M. S. Gaines. 1992. Analysis of adaptation in heterogeneous landscapes: implications for  
466 the evolution of fundamental niches. *Evolutionary Ecology* 6:433–447.
- 467 Holt, R. D., and M. E. Hochberg. 2002. Virulence on the edge: a source-sink perspective. Pages 104–119  
468 *in* U. Dieckmann, J. A. J. Metz, M. W. Sabelis, and K. Sigmund, editors. *Adaptive dynamics of*  
469 *infectious diseases: in pursuit of virulence management*. Cambridge University Press, Cambridge,  
470 UK.
- 471 Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous Inference in General Parametric Models.  
472 *Biometrical Journal* 50:346–363.
- 473 Jaenike, J., and I. Dombeck. 1998. General-purpose genotypes for host species utilization in a nematode  
474 parasite of *Drosophila*. *Evolution* 52:832–840.
- 475 Kassen, R. 2002. The experimental evolution of specialists, generalists, and the maintenance of diversity.  
476 *Journal of Evolutionary Biology* 15:173–190.
- 477 Kawecki, T. J. 1994. Accumulation of deleterious mutations and the evolutionary cost of being a  
478 generalist. *The American Naturalist* 144:833–838.

- 479 Kelly, D. W., R. A. Paterson, C. R. Townsend, R. Poulin, and D. M. Tompkins. 2009. Parasite spillback: A  
480 neglected concept in invasion ecology? *Ecology* 90:2047–2056.
- 481 Legros, M., and J. C. Koella. 2010. Experimental evolution of specialization by a microsporidian parasite.  
482 *BMC Evolutionary Biology* 10:159.
- 483 Lenormand, T. 2002. Gene flow and the limits to natural selection. *Trends in Ecology and Evolution*  
484 17:183–189.
- 485 Lenormand, T., N. Harmand, and R. Gallet. 2018. Cost of resistance: an unreasonably expensive concept.  
486 *Rethinking Ecology* 3:51–70.
- 487 Lievens, E. J. P., J. M. A. Perreau, P. Agnew, Y. Michalakis, and T. Lenormand. 2018. Decomposing  
488 parasite fitness reveals the basis of specialization in a two-host, two-parasite system. *Evolution*  
489 *Letters* 2:390–405.
- 490 Lievens, E. J. P., N. O. Rode, J. Landes, A. Segard, R. Jabbour-Zahab, Y. Michalakis, and T. Lenormand.  
491 2019. Long-term prevalence data reveals spillover dynamics in a multi-host (*Artemia*), multi-  
492 parasite (Microsporidia) community. *International Journal for Parasitology*.
- 493 Magalhães, S., E. Blanchet, M. Egas, and I. Olivieri. 2009. Are adaptation costs necessary to build up a  
494 local adaptation pattern? *BMC Evolutionary Biology* 9:182.
- 495 Magalhães, S., A. Cailleau, E. Blanchet, and I. Olivieri. 2014. Do mites evolving in alternating host plants  
496 adapt to host switch? *Journal of Evolutionary Biology* 27:1956–1964.
- 497 Martin, G., and T. Lenormand. 2015. The fitness effect of mutations across environments: Fisher's  
498 geometrical model with multiple optima. *Evolution* 69:1433–1447.
- 499 Messina, F. J., and S. L. Durham. 2015. Loss of adaptation following reversion suggests trade-offs in host  
500 use by a seed beetle. *Journal of Evolutionary Biology* 28:1882–1891.
- 501 Nidelet, T., and O. Kaltz. 2007. Direct and correlated responses to selection in a host-parasite system:  
502 testing for the emergence of genotype specificity. *Evolution* 61:1803–1811.
- 503 Poullain, V., S. Gandon, M. A. Brockhurst, A. Buckling, and M. E. Hochberg. 2008. The evolution of  
504 specificity in evolving and coevolving antagonistic interactions between a bacteria and its phage.  
505 *Evolution* 62:1–11.
- 506 Prenter, J., C. MacNeil, J. T. A. Dick, and A. M. Dunn. 2004. Roles of parasites in animal invasions. *Trends*  
507 *in Ecology and Evolution* 19:385–390.
- 508 R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical  
509 Computing, Vienna, Austria.

- 510 Ravigne, V., U. Dieckmann, and I. Olivieri. 2009. Live where you thrive: joint evolution of habitat choice  
511 and local adaptation facilitates specialization and promotes diversity. *The American Naturalist*  
512 174:E141–E169.
- 513 Reeve, M. R. 1963. The filter-feeding of *Artemia* I. In pure cultures of plant cells. *The Journal of*  
514 *Experimental Biology* 40:195–205.
- 515 Refardt, D., and D. Ebert. 2006. Quantitative PCR to detect, discriminate and quantify intracellular  
516 parasites in their host: an example from three microsporidians in *Daphnia*. *Parasitology* 133:11–8.
- 517 Remold, S. 2012. Understanding specialism when the Jack of all trades can be the master of all.  
518 *Proceedings of the Royal Society of London, Series B* 279:4861–9.
- 519 Rode, N. O., J. Landes, E. J. P. Lievens, E. Flaven, A. Segard, R. Jabbour-Zahab, Y. Michalakis, P. Agnew, C.  
520 P. Vivarès, and T. Lenormand. 2013a. Cytological, molecular and life cycle characterization of  
521 *Anostracospora rigaudi* n. g., n. sp. and *Enterocytoospora artemiae* n. g., n. sp., two new  
522 microsporidian parasites infecting gut tissues of the brine shrimp *Artemia*. *Parasitology* 140:1168–  
523 85.
- 524 Rode, N. O., E. J. P. Lievens, A. Segard, E. Flaven, R. Jabbour-Zahab, and T. Lenormand. 2013b. Cryptic  
525 microsporidian parasites differentially affect invasive and native *Artemia* spp. *International Journal*  
526 *for Parasitology* 43:795–803.
- 527 Ronce, O., and M. Kirkpatrick. 2001. When sources become sinks: migrational meltdown in  
528 heterogeneous habitats. *Evolution* 55:1520–1531.
- 529 Schmid-Hempel, P. 2011. *Evolutionary parasitology: the integrated study of infections, immunology, and*  
530 *genetics*. Oxford University Press, Oxford, UK.
- 531 Smith, J. E. 2009. The ecology and evolution of microsporidian parasites. *Parasitology* 136:1901–1914.
- 532 Streicker, D. G., A. Fenton, and A. B. Pedersen. 2013. Differential sources of host species heterogeneity  
533 influence the transmission and control of multihost parasites. *Ecology Letters* 16:975–984.
- 534 Therneau, T. M. 2014. *A Package for Survival Analysis in S*.
- 535 Turner, P. E., and S. F. Elena. 2000. Cost of host radiation in an RNA virus. *Genetics* 156:1465–1470.
- 536 Woolhouse, M. E. J., L. H. Taylor, and D. T. Haydon. 2001. Population Biology of Multihost Pathogens.  
537 *Science* 292:1109–1112.
- 538 Yourth, C. P., and P. Schmid-Hempel. 2006. Serial passage of the parasite *Crithidia bombi* within a colony  
539 of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proceedings of the Royal Society*  
540 *of London, Series B* 273:655–659.
- 541

542 **TABLES**

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

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

545

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

Parasite line	Passage	Details
<i>A. rigaudi</i> × 'A. f. host' – Repl. 1	<u>P6</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added
<i>A. rigaudi</i> × 'A. f. host' – Repl. 2	P5 → P6	$\leq 5$ P6 hosts survived; 5 hosts added
	<u>P6</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added
<i>A. rigaudi</i> × 'A. f. host' – Repl. 3	<u>P6</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added
<i>E. artemiae</i> × 'A. f. host' – Repl. 1	P5 → P6	$\leq 5$ P6 hosts survived; 5 hosts added
	<u>P6</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added
<i>E. artemiae</i> × 'A. f. host' – Repl. 2	P4 → P5	0 P5 hosts survived; transmission repeated
	<u>P6</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added
<i>E. artemiae</i> × 'A. f. host' – Repl. 4	P4 → P5	$\leq 5$ P5 hosts survived; 5 hosts added
	P5 → P6	0 P6 hosts survived (three repetitions); eventually skipped
	<u>P5</u> → P7	$\leq 5$ P7 hosts survived; 5 hosts added

550

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

Parasite species/Treatment	Replicate	Used?	Notes
<i>A. rigaudi</i> × 'A. f. host'	1	Y	
	2 revived	Y	Lost, but revived from P6 backup sample.
	3	N	Lost.
	4	Y	
<i>A. rigaudi</i> × 'Alternating hosts'	1	Y	
	2	Y	
	3	Y	
	4	N	Lost.
<i>A. rigaudi</i> × 'A. p. host'	1	Y	
	2	Y	
	3	Y	
	4	Y	
<i>E. artemiae</i> × '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.
<i>E. artemiae</i> × 'Alternating hosts'	1	Y	Attempted revival from P6 was unsuccessful.
	2 + 2 revived	Y	Revived from P6 to compare virulence.
	3	Y	
	4	Y	
<i>E. artemiae</i> × '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
<b><i>A. rigaudi</i></b>	
<i>A. franciscana</i>	66 %
<i>A. parthenogenetica</i>	70 %
<b><i>E. artemiae</i></b>	
<i>A. franciscana</i>	68 %
<i>A. parthenogenetica</i>	34 %

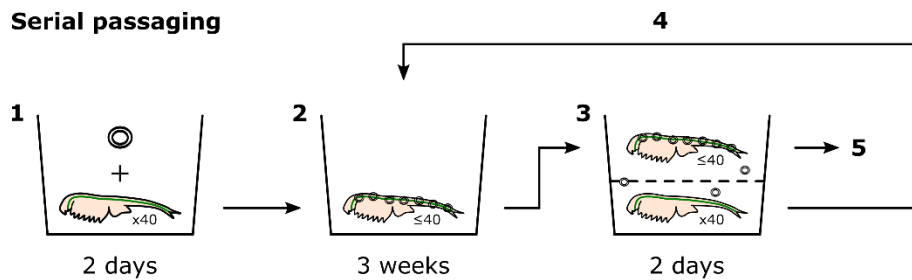
555

556

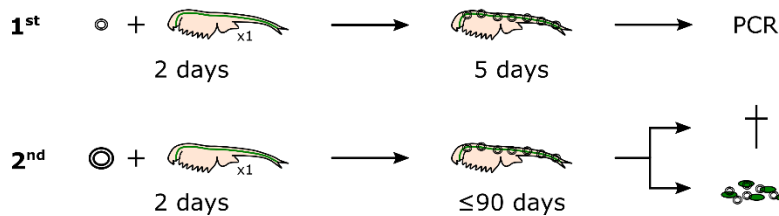
557 **FIGURES**

558 **Figure 1.** Experimental evolution protocol. **Serial passages:** [1] Passage 1 (P1): a group of 40 uninfected hosts was  
559 exposed to a saturating dose of stock *A. rigaudi* or *E. artemiae* spores, and [2] the infections were allowed to  
560 incubate. [3] Passaging (P1 → P2): the infection was transmitted naturally, by placing the surviving P1 hosts in a  
561 strainer above a new group of uninfected hosts. [4] The incubation and passaging steps were repeated for P2-P10.  
562 [5] After passaging, the surviving old hosts were counted (P1-P10) and used to estimate the population size of the  
563 parasite (P1, P4, P7), produce backup spore samples (P6), or produce the spores for the final assays (P10). **Final**  
564 **assays:** [1<sup>st</sup>] An uninfected host was exposed to a low dose of evolved *A. rigaudi* or *E. artemiae* spores, and PCR-  
565 tested for the presence of the microsporidian after a short incubation period. [2<sup>nd</sup>] An uninfected host was  
566 exposed to a saturating dose of evolved *A. rigaudi* or *E. artemiae* spores, and mortality and spore production were  
567 tracked for 90 days.

**Serial passaging**



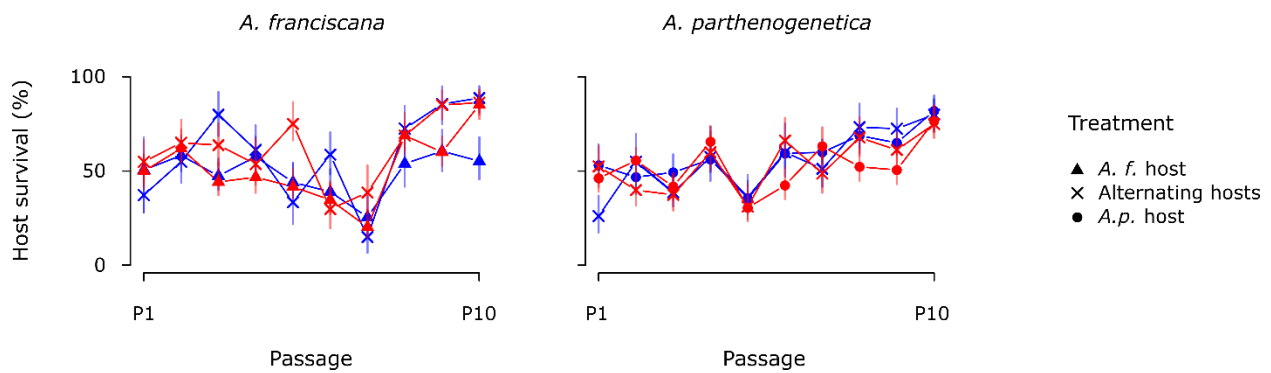
**Final assays**



568

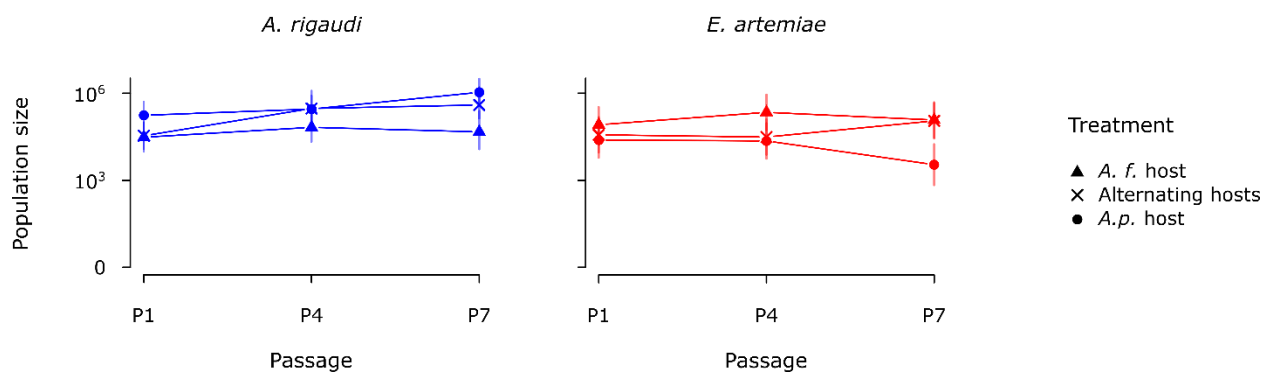
569

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

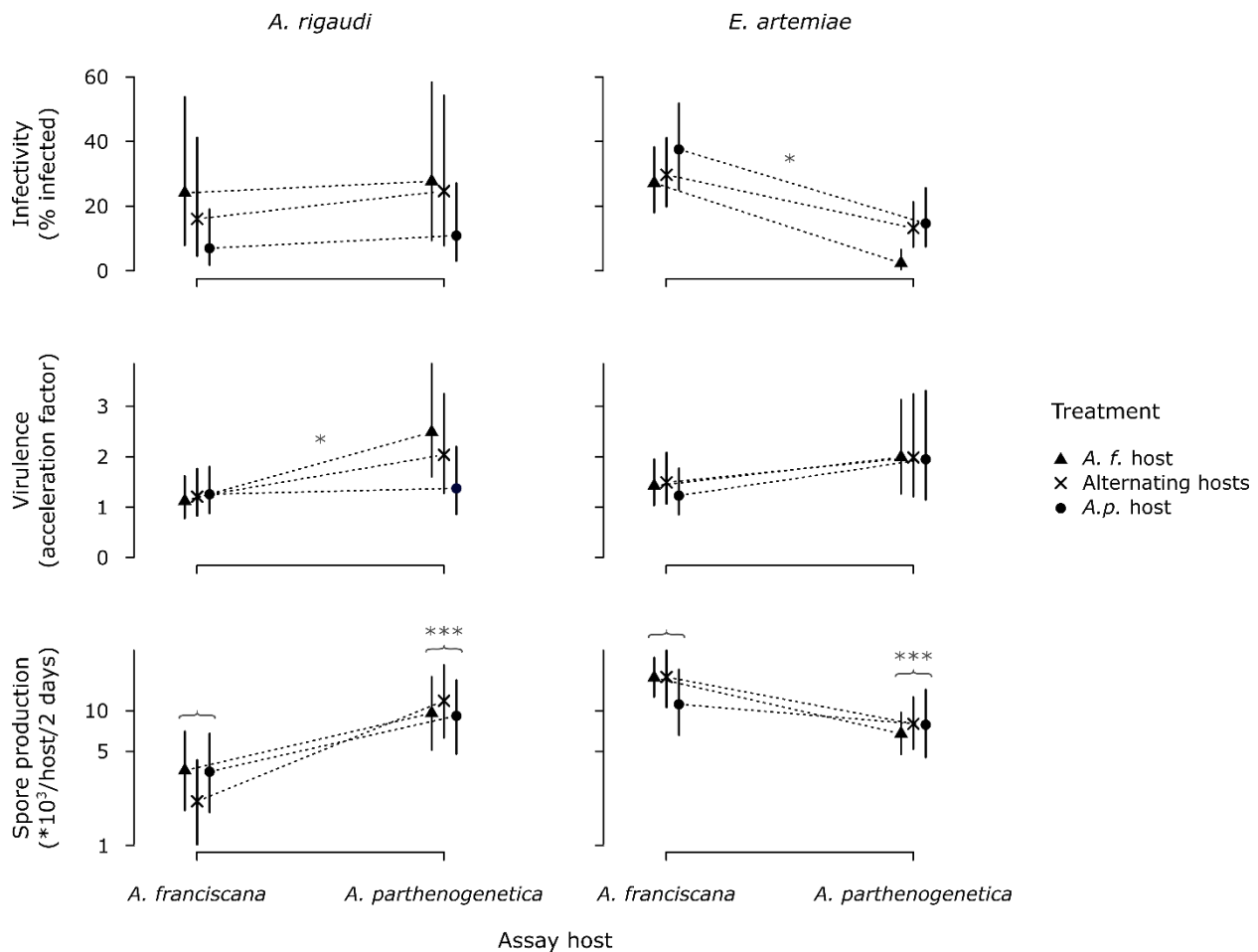
575 **Figure 3.** Parasite population size, as estimated for each passaging line (*ln* scale). Colors denote the parasite  
576 treatment (blue, *A. rigaudi*; red, *E. artemiae*); symbols denote the passaging treatment (triangles, 'A. f. host';  
577 crosses, 'Alternating hosts'; circles, 'A. p. host');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



579

580

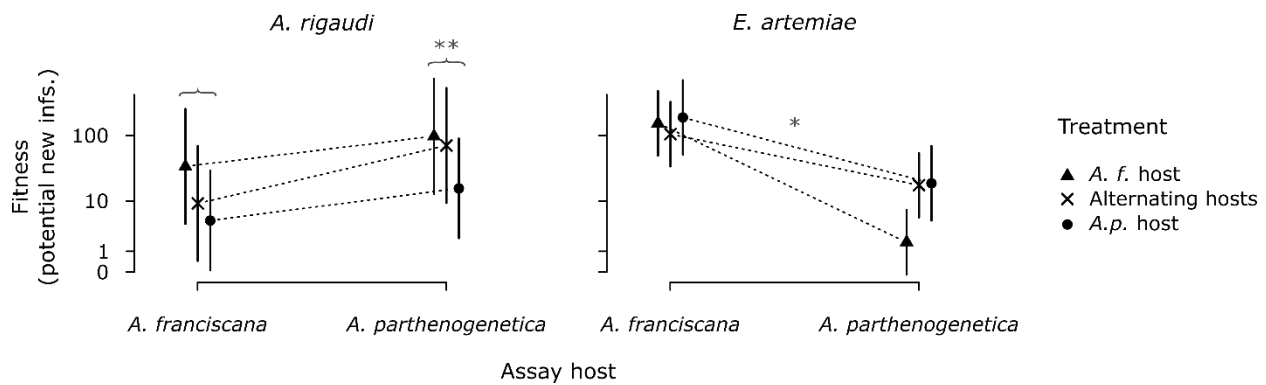
581 **Figure 4.** Infectivity, virulence, and spore production during the final assays. Infectivity is the percentage of hosts  
 582 infected during the first assay. Virulence and spore production were measured in the second assay: virulence is the  
 583 acceleration factor (the ratio of time-until-death) 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.



588

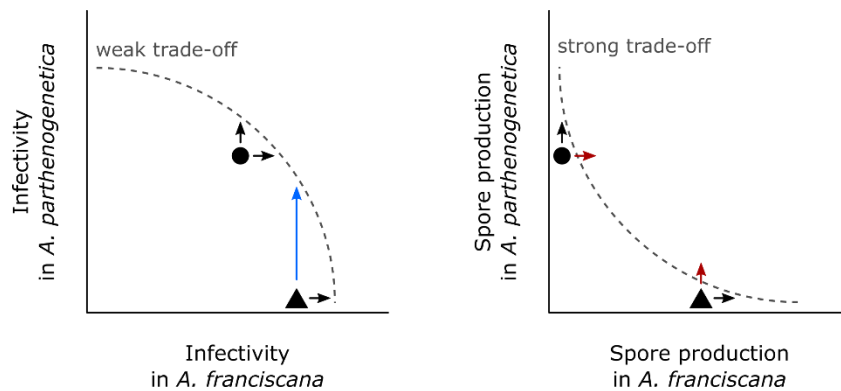
589

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



594

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



600