DECOMPOSING PARASITE FITNESS IN A TWO-HOST, TWO-PARASITE SYSTEM REVEALS THE UNDERPINNINGS OF PARASITE SPECIALIZATION

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ABSTRACT

The ecological specialization of parasites – whether they can obtain high fitness on very few or very many different host species – is a determining feature of their ecology. In order to properly assess specialization, it is imperative to measure parasite fitness across host species; to understand its origins, fitness must be decomposed into the underlying traits. In this study, we quantified the infectivity, virulence, and transmission rate of two parasites, the horizontally transmitted microsporidians *Anostracospora rigaudi* and *Enterocytospora artemiae*, in their natural hosts, the brine shrimp *Artemia parthenogenetica* and *Artemia franciscana*. We performed experimental infections, and tracked the production of spores, the growth, the reproduction, and the survival of each infected host. Our results demonstrate that each parasite performs well on one of the two host species (*A. rigaudi* on *A. parthenogenetica*, and *E. artemiae* on *A. franciscana*), and poorly on the other. This partial specialization is driven by high infectivity and transmission rates in the preferred host, and is associated with maladaptive virulence and large costs of resistance in the other. Our study represents a rare empirical contribution to the study of parasite evolution in multi-host systems, highlighting the value of decomposing parasite fitness when investigating parasite specialization.

KEYWORDS

Artemia

Microsporidians

Multi-host

Multi-parasite

Parasite fitness

Host specificity

Fecundity compensation

Resistance

INTRODUCTION

The degree of specialization is a key trait of parasite species. Even within clades, parasites can range from extremely specific (infecting only one host species) to widely generalist (infecting tens of host species) (Poulin and Keeney, 2008). Specialization is an evolved trait (Cenzer, 2016; e.g. Desdevises et al., 2002; Johnson et al., 2009; Tanaka et al., 2007), which in turn has an important influence on the future evolution of the parasite. Generalist parasites are more likely to survive perturbations in the host community and to achieve host switches (Agosta et al., 2010; Cleaveland et al., 2001), while specialist parasites are more likely to coevolve tightly with their hosts (Kawecki, 1998).

The 'ecological specialization' (Futuyma and Moreno, 1988) of parasites should be distinguished from the standard concepts 'host range' and 'host specificity' (sensu Lymbery, 1989). Neither host range – the number of host species in which a parasite occurs – nor host specificity – host range weighted by infection intensity or by phylogenetic relationships between the hosts – account for the existence of host species that barely contribute to the parasite's transmission. Such 'spillover' hosts (sensu Fenton et al., 2015) can readily become infected, and may even transmit the parasite, but cannot maintain a persistent parasite population (the parasite's population growth is below one). Therefore, unless there is constant transmission from suitable hosts, infection in the spillover hosts quickly dies out ("dead-end" and "stuttering chain" dynamics, Viana et al., 2014). In essence, these are ecological source-sink dynamics; the unsuitable hosts fall outside the fundamental niche of the parasite (Pulliam, 1988). Therefore, hosts that cannot maintain a parasite population should not contribute equally to its classification as an ecological generalist or specialist (Poulin and Keeney, 2008). To assess ecological specialization, the parasite's fitness must be studied in all the infected host species. This typically requires the establishment of detailed epidemiological models (Fenton et al., 2015; e.g. Rhodes et al., 1998) or the experimental measurement of parasite fitness (Ahonen et al., 2006; Auld et al., 2017; e.g. Jaenike and Dombeck, 1998).

A second step is to understand why parasite fitness varies across hosts. The fitness of infections emerges from a suite of parasite- and host-determined traits, including infectivity, exploitation of host resources, virulence, immune evasion, and transmission success. The nature of these traits has important consequences for a parasite: functional correlations between traits within a host species (Alizon and Michalakis, 2015; Alizon et al., 2009; Hall et al., 2017; Walther and Ewald, 2004), or between a trait in different host species (Futuyma and Moreno, 1988; Via and Hawthorne, 2002) can constrain its evolutionary trajectory. They also determine the source of the parasite's maladaptation to spillover hosts (Woolhouse et al., 2001). For parasites, this has been best studied with regards to virulence and transmission, and that mostly in single-host systems (de Roode et al., 2008; Doumayrou et al., 2012; e.g. Dwyer et al., 1990; Fraser et al., 2007). Studies that decompose fitness into multiple underlying traits in multi-host parasites are rare (Rigaud et al., 2010)(e.g. Agudelo-Romero et al., 2008; Auld et al., 2017).

Here, we examine specialization and its composite traits in a natural multi-host, multi-parasite system. In the saltern of Aigues-Mortes, France, two species of brine shrimp occur in sympatry: a native parthenogenetic clade, *Artemia parthenogenetica*, and an introduced sexual species, *Artemia franciscana* (Amat et al., 2005). Both *Artemia* species are parasitized by the microsporidians *Anostracospora rigaudi* and *Enterocytospora artemiae*. These parasites infect the gut epithelium, transmitting infection horizontally through spores released with the faeces (Nicolas Olivier Rode et al., 2013; Rode et al., 2013). Since the saltern lacks spatial structure (cf. Fels, 2006; Nougué et al., 2015), the pool of microsporidian spores is shared between *A. franciscana* and *A. parthenogenetica*. Although the potential rates of inter-specific transmission should therefore be high, and both *A.*

rigaudi and *E. artemiae* commonly infect either host species, the two microsporidians appear to be somewhat specialized: *A. rigaudi* is always more prevalent in *A. parthenogenetica*, and is also dependent on this species to maintain itself in the host community; in contrast, *E. artemiae* is consistently more infectious to and more prevalent in *A. franciscana* (Lievens et al., n.d.). Historically, the association of *A. parthenogenetica* and *A. rigaudi* predates the introduction of *A. franciscana* (in 1970, Rode et al., 2013), while *A. franciscana* is also infected by *E. artemiae* in its native range (Rode et al., 2013). It is not known whether *E. artemiae* was also present in France before the introduction of *A. franciscana*, whether it was co-introduced, or whether it arrived independently afterwards.

We studied the infectivity, virulence, and transmission of *A. rigaudi* and *E. artemiae* in each of their hosts. We confirm experimentally that while both microsporidians can complete their life cycle in the two host species, neither is a complete generalist. Rather, *A. rigaudi* is largely specialized on *A. parthenogenetica*, while *E. artemiae* is largely specialized on *A. franciscana*. Further, we show that the lower fitness of the two parasites in their non-specialized hosts was caused by a reduction in infectivity and transmission rate (in both cases), combined with a suboptimal degree of virulence (too low for *E. artemiae*; too high for *A. rigaudi*). Overall, by decomposing and comparing the two microsporidians' fitness in their shared hosts, we were able to draw general conclusions about the origin of specialization in this system.

METHODS

We performed two experiments to investigate the life history and virulence of the microsporidians *A. rigaudi* and *E. artemiae* in their *Artemia* hosts. First, we used dose-response tests to quantify infectivity in each host-parasite combination. Second, we did a large-scale experimental infection experiment, tracking host growth, mortality, and reproduction, as well as parasite transmission, over a period of two months. These results allowed us to estimate the virulence and fitness of each parasite on each host.

Experimental conditions

The *Artemia* used in both experiments were raised in the lab in parasite-free conditions. *A. franciscana* were hatched from dormant cysts sampled from the saltern of Aigues-Mortes, France, and stored in dry conditions at 4 °C. We used three batches of cysts, sampled at the sites Caitive Nord or Caitive Sud in October 2013 or 2014. Cysts were hatched following the protocol described by Lievens et al. (2016). *A. parthenogenetica* were collected as live larvae from a mix of clones. The *A. parthenogenetica* clones were started by females collected in Aigues-Mortes, who were allowed to multiply and produce cysts in the lab; those cysts were then hatched to produce parasite-free stock lines. All *Artemia* were maintained at 23 ± 1 °C, in a parasite-free 90 ppt saline medium produced by diluting concentrated, autoclaved brine (Camargue Pêche, France) with deionized water. *Artemia* were fed *ad libitum* with freeze-dried microalgae (*Tetraselmis chuii*, Fitoplankton marino, Spain) dissolved in deionized water. Experimental conditions matched the cultivation conditions, except that feeding was regulated (see below).

We created stocks of *A. rigaudi* and *E. artemiae* for use in the experiment by combining infected *Artemia* from various sites in Aigues-Mortes between October 2014 and March 2015. We added new infected hosts to the stocks whenever we found field populations that were heavily infected with either *A. rigaudi* or *E. artemiae*. We also regularly added uninfected, lab-bred *Artemia* to help

123 maintain the infection. We selected both infected *A. franciscana* and infected *A. parthenogenetica*
124 from the field, and maintained each stock population on a mix of *A. franciscana* and *A.*
125 *parthenogenetica* hosts (n_{hosts} at any given time = ~20-~50 per microsporidian species). Thus, our
126 stocks contained a mix of spores from different field sites and times, collected from and propagated
127 on both host species.

128 **Spore collection and quantification**

129 To produce the inocula for Experiments 1 and 2, we collected spores from the lab stocks of *A. rigaudi*
130 and *E. artemiae* described above. The stock hosts were kept in large separating funnels, so that their
131 feces (containing spores) settled down into the funnel's tube and could be collected easily. For our
132 experiments, we collected feces produced over 20-hour periods (feces suspended in ~15 mL).
133 Because fecal aggregates can trap spores and skew concentration estimates, we homogenized the
134 fecal solutions by dividing them into 1.2 mL Qiagen Collection Microtubes, adding two 4 mm
135 stainless steel beads to each tube, and shaking them at 30 Hz for 30 s. Once homogenized, the fecal
136 solutions were recombined to their original volume. To quantify the spore concentration in the fecal
137 solutions, we took 1 mL subsamples and added 10 µL 1X Calcofluor White Stain (18909 Sigma-
138 Aldrich, USA) to each. After staining for 10 min, we rinsed the subsamples by centrifuging them for 8
139 min at 10 000 g, replacing 910 µL of the supernate with 900 µL deionized water and vortexing well.
140 We then concentrated the subsamples to 20X by repeating the centrifugation step and removing
141 950 µL of the supernate. Finally, we estimated the concentration by counting the number of spores
142 in 0.1 µL on a Quick Read counting slide (Dominique Dutscher) under a Zeiss AX10 fluorescence
143 microscope (10x 40x magnification; excitation at 365 nm; Zeiss filter set: 62 HE BFP + GFP + HcRed
144 shift free (E)). We repeated the counts twice (for Experiment 2) or thrice (for Experiment 1); the
145 spore concentration per µL in the unconcentrated fecal solutions was then equal to (mean of the
146 spore counts*10)/20. Finally, we added 90 ppt clean saline medium to the homogenized fecal
147 solutions until the correct concentration for inoculation was reached.

148 **Experiment 1: Infectivity**

149 *Experimental design and execution*

150 Previous papers studied the infectivity of *A. rigaudi* and *E. artemiae* using single, uncontrolled spore
151 doses (Lievens et al., n.d.; Nicolas Olivier Rode et al., 2013). Here, we quantified infectivity more
152 precisely by exposing individual *A. parthenogenetica* and *A. franciscana* to a range of controlled
153 spore doses and measuring the proportion of infected individuals.

154 We exposed experimental hosts to doses of 0, 400, 800, 1 600, 3 200 and 6 400 spores per individual.
155 To ensure hosts ingested all spores, each host was first exposed in a highly-concentrated medium:
156 individuals were placed in 2 mL Eppendorf tubes with 0.45 mL spore solution, 1 mL extra brine and
157 0.25 mL algal solution ($3.4*10^9$ *T. chuii* cells/L deionized water). After two days, hosts were
158 transferred to 40 mL glasses containing 20 mL brine and the infection was allowed to incubate for
159 three more days; hosts were fed a total of 1 mL algal solution over the three days. Surviving hosts
160 were then sacrificed and tested for the presence of *A. rigaudi* or *E. artemiae* by PCR (following
161 Nicolas Olivier Rode et al., 2013). Treatments were replicated 20 times, except when spore
162 availability was limiting (*E. rigaudi* on *A. parthenogenetica*: 16, 8 and 4 replicates for the doses 400,
163 3 200 and 6 400 spores per individual, respectively). All hosts were ~4 weeks old and measured
164 between 5 and 8 mm; *A. franciscana* hosts were mixed males and females.

165 *Statistical analyses*

166 To analyze the dose-response curves, we used four-parameter log-logistic modeling in R (R Core
167 Team, 2014; package drc, Ritz and Strebig, 2005). In these models, the four parameters determining
168 the shape of the sigmoidal curve are: the lower limit (set to 0 in our case), the upper limit, the slope
169 around the point of inflection, and the point of inflection (which here is the same as the ED_{50}). The
170 (binomial) response variable was the number of individuals that were infected vs. uninfected.
171 Because we did not perform the *A. parthenogenetica* and *A. franciscana* experiments at the same
172 time, we could not control for environmental effects. Thus, we simply tested if the dose-response
173 curves for *A. rigaudi* and *E. artemiae* were different within each host species. To do this, we fit
174 models that did or did not include a 'microsporidian species' effect and compared the two using a
175 likelihood ratio test. If the effect was significant, we went on to compare the parameters of the two
176 resulting curves ('compParm' function in the drc package).

177 **Experiment 2: Virulence and transmission**

178 *Experimental design and execution*

179 To quantify the virulence and transmission rates of *A. rigaudi* and *E. artemiae*, we experimentally
180 infected individual *Artemia* with controlled spore doses. We then tracked their survival, growth,
181 reproductive output, and spore production over a two-month period. We also quantified host-to-
182 host transmission at two time points.

183 *A. franciscana* males, *A. franciscana* females, and *A. parthenogenetica* females were divided into
184 three treatments: 'Controls', 'Exposure to *A. rigaudi*, and 'Exposure to *E. artemiae*', which were
185 replicated as permitted by spore and host availability (Table 1). *A. franciscana* hosts were subdivided
186 into three blocks, determined by their origin: Caitive Nord 2013, Caitive Nord 2014, or Caitive Sud
187 2014. *A. parthenogenetica* hosts were subdivided into two blocks, determined by the age of their
188 batch: 34 ± 2 or 26 ± 2 days (because the relative contribution of the different clones to the batches
189 was not controlled, the genotype frequencies of these groups could differ). All hosts were subadults
190 (adult body plan but sexually immature). All *A. franciscana* were aged 38 ± 1 days and measured 4.5
191 or 5.0 mm; *A. parthenogenetica* measured 6.5, 7.0 or 7.5 mm. Size classes were evenly distributed
192 across blocks and treatments.

193 **Table 1. Number of replicates for the different treatments and blocks.** See 'Methods – Experiment 2' for
194 more information.

Treatment:	**Exposure to *A. rigaudi***		**Exposure to *E. artemiae***			**Controls**	
[spore dose]	[3 000 sp/i]		[2 500 sp/i]		[10 000 sp/i]		
A. franciscana	**86 ♂**	**86 ♀**	**132 ♂**	**132 ♀**		**120 ♂**	**120 ♀**
Origin: Caitive Nord 2013	26 ♂	26 ♀	72 ♂	72 ♀		60 ♂	60 ♀
Origin: Caitive Nord 2014	30 ♂	30 ♀	30 ♂	30 ♀		30 ♂	30 ♀
Origin: Caitive Sud 2014	30 ♂	30 ♀	30 ♂	30 ♀		30 ♂	30 ♀
A. parthenogenetica		**96 ♀**		**96 ♀**	**33 ♀**		**96 ♀**
Batch: 34 ± 2 days old		48 ♀		48 ♀	18 ♀		48 ♀
Batch: 26 ± 2 days old		48 ♀		48 ♀	15 ♀		48 ♀

195

196 We exposed experimental hosts to spore doses designed to be comparable while maximizing
197 infection rate (see results of Experiment 1): 3 000 spores/individual for *A. rigaudi* and 2 500
198 spores/individual for *E. artemiae*. Because *A. parthenogenetica* had low infection rates with *E.*

199 *artemiae*, a separate set of *A. parthenogenetica* was infected with 10 000 *E. artemiae* spores per
200 individual (Table 1). To ensure hosts ingested all spores, each host was exposed in a highly-
201 concentrated medium over a two-day period: individuals were placed in 2 mL Eppendorf tubes with
202 0.37 mL spore solution and 1.25 mL brine containing $2.6*10^6$ *T. chuii* cells.

203 After exposure (= on day 1 of the experiment), individuals were transferred to open tubes, which
204 rested upright in 40 mL plastic cups containing 20 mL of brine. The lower end of the tube was fitted
205 with a 1x1 mm net. The netting prevented experimental (adult) individuals from swimming to the
206 bottom of the cup, while allowing spores, feces, and offspring to pass through; this limited
207 secondary infections from a host's own feces. Cups were randomly placed in trays, which were
208 routinely rotated to standardize effects of room placement. Water was changed every five days.
209 Hosts were fed 0.5 mL algal solution daily ($2.6*10^9$ *T. chuii* cells/L deionized water); this feeding
210 regime corresponds to half of the maximum ingestion rate of an adult *Artemia* (Reeve, 1963) and
211 has been shown to reveal energetic trade-offs (Rode et al., 2011). We ended our experiment after 60
212 days, at which point surviving individuals were sacrificed and tested for infection by PCR (following
213 Nicolas Olivier Rode et al., 2013).

214 To quantify the effects of infection on the hosts, we tracked the growth, survival, and reproduction
215 of the experimental individuals. Body length was recorded on days 30 and 60. Survival was recorded
216 daily; dead individuals were tested for infection by PCR (following Nicolas Olivier Rode et al., 2013).
217 We did not track reproduction for males, because male reproductive success is heavily influenced by
218 the female partner (e.g. female clutch size). For females, measures of reproductive success were
219 recorded daily, including date of sexual maturity (first detection of a fully-formed ovisac or of yolk
220 accumulation in oocytes, Metalli and Ballardin, 1970), clutch date, clutch type, and clutch size.
221 *Artemia* females are iteroparous, producing on average one clutch per five days (Bowen, 1962;
222 Metalli and Ballardin, 1970). Clutches may be of two types: live larvae ('nauplii'), or dormant
223 encysted embryos ('cysts'). Neonatal nauplii are barely visible to the eye and have high death rates.
224 For ease of measurement, therefore, clutches of nauplii were counted five days after sighting.
225 During these five days nauplii were in competition for resources with their mother (plus an
226 additional male if *A. franciscana*, see below). However, mothers were removed at each water
227 change, which could happen before the clutch had reached the five-day mark. In these cases, we
228 placed a new tube containing one (or two, if *A. franciscana*) adult male *Artemia* above the nauplii to
229 ensure the same level of food competition.

230 While *A. parthenogenetica* females reproduce in isolation, *A. franciscana* females need to be
231 fertilized before each clutch (Bowen, 1962). We therefore added mature *A. franciscana* males from
232 parasite-free lab stocks to each tube containing an *A. franciscana* female. To prevent cross-
233 contamination between the male and the female, exposed males were removed and new uninfected
234 males added every five days (five-day estimate based on infection detection time as found by
235 Nicolas Olivier Rode et al., 2013). Male *Artemia* mate-guard by clasping females around the
236 abdomen (Bowen, 1962), and forcible removal may be harmful to both partners. To avoid this, males
237 found mate-guarding on the fifth day were given up to two extra days with the female, after which
238 they were forcibly removed. Couples were fed twice the individual food allocation.

239 To estimate parasite fitness, we estimated spore production at regular points throughout the
240 experiment. To do this, we collected 1 mL of feces (containing parasite spores) from every
241 experimentally infected host at every water change. Samples were stored in 1.2 mL Qiagen
242 Collection Microtubes and refrigerated until the spore concentration could be quantified. To
243 measure the spore concentration, we homogenized and stained each sample as described above,
244 with minor differences in the centrifugation steps (16 min at 5 000 g) and the final concentration

245 (concentrated to 14.3X by removing 930 µL of the supernate). Spores were counted once per
246 sample, as described above. Because counting spores is labor-intensive, we restricted our efforts to
247 the feces samples collected on days 15, 30, 45 and 60.

248 We also investigated the host-to-host transmission success of the parasites and its relation to spore
249 production. On days 30 and 60, we allowed a subset of experimental hosts (hereafter the 'donors')
250 to infect groups of uninfected 'recipient' hosts for 24 hours. Donors were first placed with either
251 eight *A. franciscana* or eight *A. parthenogenetica* recipients; after 24 hours, the donor was removed
252 and placed with a new group of recipients of the other species. All recipient hosts were taken at
253 random from uninfected lab stocks of varying ages and sizes (min = 4 mm, max = 10 mm). The donor
254 host was separated from the recipients by a 1x1 mm net; recipients swam underneath them in 40
255 mL of brine. Infection was allowed to incubate in the recipients for six days after the donor was
256 removed; surviving recipients were then sacrificed and PCR-tested for infection (following Nicolas
257 Olivier Rode et al., 2013). The prevalence of infection in recipient individuals could then be
258 compared to the number of spores counted in the feces samples on day 30 or 60.

259 A key aspect of infection follow-up experiments is knowing which individuals were infected after
260 exposure to the parasite, and which were not. In our experiment, testing by PCR was often not
261 sufficient to determine if an individual was infected, because individuals that died before day 60
262 often had quickly decaying corpses and thus degraded DNA. We therefore considered that an
263 individual was infected if it tested positive by PCR or produced spores or transmitted the infection to
264 a recipient host. If none of these requirements were met, we considered that the individual was not
265 infected. By applying these criteria, we could be sure of the infection status for almost all individuals
266 that died on or after day 15 (the first spore collection date); for any individuals who died before day
267 15 and who tested negative by PCR, we could not exclude the possibility that they were infected.

268 *Statistical analyses: species-level effects*

269 We analyzed the results of this experiment in two major parts. First, we examined the virulence of
270 infections (effect of the parasite on host survival, growth, reproduction, and overall fitness).
271 Importantly, we only analyzed virulence once we could be certain of an individual's infection status.
272 To do this, we excluded all individuals that died before day 15 (see above), and only compared
273 infected with control individuals. To make sure that we were not missing important events occurring
274 before this cutoff, we repeated all statistical models for exposed vs. control individuals that died
275 before day 15. Second, we used the spore production results to analyze parasite transmission (spore
276 production rate, transmission success, infectiousness) and to estimate overall parasite fitness.

277 An overview of the analyses is given in Table 2; a detailed description can be found in the
278 Supplementary Material. Below, we describe only those response variables that are not intuitive.

279 To estimate the infectiousness of a single spore, we used the results of the transmission assay. We
280 assumed that the establishment of microsporidian infections follows an independent-action model
281 with birth-death processes. This model assumes that a parasite population grows in the host until it
282 reaches an infective threshold, at which point the infection is considered to be established (Schmid-
283 Hempel, 2011, pp. 225–6). In our assay, we considered that an infection was established when we
284 could detect it; in other words, the infective threshold corresponded to the threshold for PCR
285 detection (estimated at ~1 000 spores inside the host's body, unpublished data). In these models,
286 the probability per spore to start an infection, *p*, is equal to $-\ln\left(\frac{noninfected}{exposed}\right)/D$ where *D* is the
287 spore dose (Schmid-Hempel, 2011, pp. 225–6). In our transmission assay, *D* can be approximated by
288 the number of spores in the fecal sample taken from the donor at the start of the assay (= spore

Table 2. Overview of statistical analyses. See Supplementary Material for details.

Tested variable	Statistical models and tests	Fixed-effect terms in the full model	Random/Frailty terms
Virulence of infections: *A. franciscana* and *A. parthenogenetica* analyzed separately			
Survival	Survival models [1] + LRT + Dunnett p.-h.	Treatment [2], Sex (*A. f.*), Size class, double interactions	Origin (*A. f.*), Batch (*A. p.*)
Growth			
Timing of growth	Paired t-tests	Size on days 1 vs. 30 & 30 vs. 60	NA
Growth between days 1 & 30	LMM + LRT + Dunnett p.-h.	Treatment [2] * Sex (*A. f.*) * Size class	Origin (*A. f.*), Batch (*A. p.*)
Reproduction			
Time until sexual maturity	Survival models [1] + LRT + Dunnett p.-h.	Treatment [2] * Size class	Origin (*A. f.*), Batch (*A. p.*)
Probability of producing a clutch	Bernouilli GLMM + LRT + Dunnett p.-h.	Treatment [2] * Size class	Origin (*A. f.*), Batch (*A. p.*)
Rate of offspring production [3,4,a]	LMM + LRT + Dunnett p.-h.	Treatment [2] * Size class	Origin (*A. f.*), Batch (*A. p.*)
Timing of offspring production [3,5,a]	Neg. binomial GLMM + LRT + Dunnett p.-h.	Treatment [2] * (Elapsed % of reproductive period + Elapsed % of reproductive period^2)	Individual, Origin (*A. f.*), Batch (*A. p.*)
Type of offspring produced [a]	Binomial GLMM + LRT + Dunnett p.-h.	Treatment [2] * Size class	Origin (*A. f.*), Batch (*A. p.*)
Fitness			
Lifetime reproductive success [3,6]	Neg. binomial hurdle regressions + LRT	Treatment [2] * Size class	
Parasite transmission and fitness: infections of *A. franciscana* and *A. parthenogenetica* analyzed together			
Spore production rate [b]			
Spore count [7,c]	Neg. binomial GLMM + LRT + Tukey p.-h.	Host sp. * Parasite sp.	Individual
Spore count [7] ~ dose [d]	Neg. binomial GLMM + LRT	Dose	Individual
Transmission success ~ spore count	Spearman's ρ correlation tests	Host-parasite combination	NA
Infectiousness of one spore, p	LMM + LRT + Tukey p.-h.	Recipient sp. * Parasite sp.	Individual
Fitness			
Lifetime transmission success	Kruskal-Wallis tests + Dunn p.-h.	Host-parasite combination [2]	NA
Asymptotic growth rate	Kruskal-Wallis tests + Dunn p.-h.	Host-parasite combination [2]	NA

Notes: [1] Survival models were parametric; the best survival distribution was chosen by AICc. [2] *A. parthenogenetica* exposed to low and high doses of *E. artemiae* treated separately. [3] Offspring could be nauplii or cysts. These two offspring types were not directly comparable: they probably require different amounts of energy to produce, and we allowed mortality to occur before counting nauplii. To account for this, we repeated the tests with nauplii weighted twice, equally, or half as much as cysts, and based our conclusions on the overall pattern. [4] Rate of offspring production = total number of offspring / length of the reproductive period. The length of the reproductive period was the difference between the date of death (or censoring) and the date of sexual maturity. [5] Modeled as clutch size as a function of the elapsed proportion of the reproductive period. The reproductive period started at sexual maturity and ended at death (or censoring). [6] LRS calculated as the total number of offspring produced over the study period. [7] Spore count = the number of spores counted in the fecal sample; we did not transform the spore count to spores/mL (≈ spore count * 700) to avoid skewing the error distribution.

Subsets: [a] Only for females that produced at least 1 clutch. [b] Analyzed for infected individuals only. [c] Excluded *A. p.* exposed to high doses of *E. artemiae*. [d] Only for *A. p.* infected with *E. artemiae*.

Abbreviations: GLMM, generalized linear mixed models. LMM, linear mixed models. LRT, likelihood ratio testing. p.-h., post-hoc tests. *A. f.*, *A. franciscana*. *A. p.*, *A. parthenogenetica*. sp., species. *, interactions between the factors were included. NA, not applicable.

count transformed to spores/mL, or * 700), divided by 5*8 = 40 (fecal samples accumulated over a 5-day period but we only exposed recipients for one day; the inoculum was shared amongst 8 recipients). We calculated a value of *p* for every replicate in the transmission assay.

For each infection we used two measures of spore production as proxies for parasite fitness. First, we calculated a proxy for the 'lifetime transmission success': we summed the number of spores in the fecal samples taken on days 15, 30, 45 and 60 for each infection, then corrected this cumulative spore count by *p*, the average infectiousness of a single spore in a given host-parasite combination (as calculated above). Second, we calculated an asymptotic growth rate by computing the dominant eigenvalue of a standard Leslie matrix,

$$\begin{bmatrix} 0 & n_{15} * p & n_{30} * p & n_{45} * p & n_{60} * p \\ s_{15} & 0 & 0 & 0 & 0 \\ 0 & s_{30} & 0 & 0 & 0 \\ 0 & 0 & s_{45} & 0 & 0 \\ 0 & 0 & 0 & s_{60} & 0 \end{bmatrix},$$

where n_i is the number of spores in the fecal sample on day *i*, *p* is the average infectiousness of a single spore in that host-parasite combination (as calculated above), and s_i describes whether the host survived until day *i* (1) or not (0). While the lifetime transmission success is a measure of the basic reproduction number R_0, which describes parasite fitness under stable endemic conditions, the asymptotic growth rate is a measure of the net population growth rate, which describes fitness under epidemic conditions (Frank, 1996; Hethcote, 2000); we included both measures because either situation can occur in the field.

Statistical analyses: individual variation in resistance

In most of the experimental host-parasite combinations, a subset of exposed hosts did not become (detectably) infected. Hereafter, we refer to these individuals as resistant, because we found *a posteriori* differences in their life history traits compared to controls and infected individuals. We analyzed the distribution of resistance across host-parasite combinations, as well as its relationship with host fitness. As above, these analyses excluded all individuals who died before infection status could be definitively determined, i.e. those that died before day 15 of the experiment.

Within each host species, we used χ^2 tests to compare the numbers of resistant and infected hosts after exposure to *A. rigaudi* and *E. artemiae*. We also used χ^2 tests to test for an effect of sex on the probability of resistance to each parasite in *A. franciscana*, and for an effect of spore dose on the probability of resistance to *E. artemiae* in *A. parthenogenetica*.

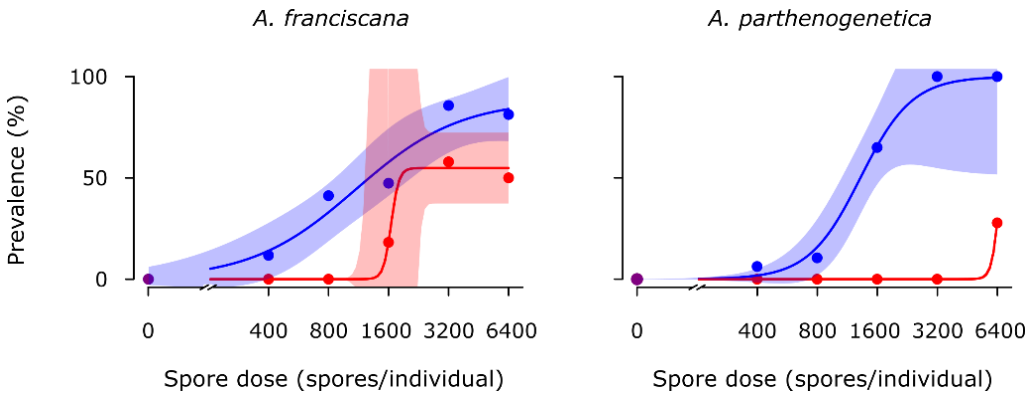
There was substantial variation in infection outcome for the combinations *A. franciscana*/'Exposure to *A. rigaudi*', and *A. parthenogenetica*/'Exposure to *E. artemiae* – low dose' (see Results). Because costs of resistance are a common aspect of host-parasite interactions (Schmid-Hempel, 2003), we investigated whether resistance was related to host fitness in these combinations. To do this, we repeated the survival and reproduction analyses described above, with an added *Resistant-Infected-Control* factor. We added or excluded this factor and its interactions with the other fixed effects, then compared all models using the corrected AIC. In this way, we investigated whether the outcome of infection explained a significant part of the variation in host traits after the experimentally manipulated factors were taken into account. If the *Resistant-Infected-Control* factor was maintained in the best models, we used contrast manipulation and AICc-based model comparison to detect how the three host categories (*Resistant*, *Infected*, *Control*) differed.

340 # RESULTS

341 ## Experiment 1: Infectivity

342 Both *A. parthenogenetica* and *A. franciscana* were more susceptible to infection with *A. rigaudi* than
343 *E. artemiae* ($\chi^2(3) \geq 20.9$, $p < 0.001$ for both; Fig. 1). For *A. franciscana*, the slopes and inflection points
344 of the two curves were not significantly different, but the upper limit was significantly higher for *A.*
345 *rigaudi* than for *E. artemiae* ($t = 2.1$, $p = 0.03$). In *A. parthenogenetica*, the infectivity of the two
346 parasites was markedly different: successful infections with *E. artemiae* required such a high spore
347 dose that the inflection point and upper limit of its curve could not be computed; its slope was not
348 significantly different to that of *A. rigaudi*. Mortality was not dose-dependent in any of the host-
349 microsporidian combinations, so we can be confident that it did not skew results (Supp. Table 1).

350 **Figure 1. Infectivity of *A. rigaudi* (blue) and *E. artemiae* (red) in *A. franciscana* (left) and *A. parthenogenetica***
351 **(right).** Points indicate the prevalence (% infected) at each dose; lines are the best fits and the shaded areas
352 represent the 95% CIs. Because the inflection point of *E. artemiae* in *A. franciscana* was poorly resolved,
353 uncertainty was high here. It was not possible to calculate a confidence interval for *E. artemiae* in *A.*
354 *parthenogenetica* due to low resolution.



355

356 ## Experiment 2: Virulence and transmission

357 Among host individuals that survived until we could be certain of their infection status (i.e. that
358 survived until at least day 15), infection rates were high (Table 3). As expected, many fewer
359 infections were detected among individuals that died before day 15. In general, infection rates in
360 Experiment 2 were considerably higher than those in Experiment 1; this was most likely because the
361 longer incubation time allowed slow-growing infections to become detectable.

362 **Table 3. Detection of infection before and after the detection threshold.** The table shows the detected
363 infection rates in individuals that survived until we could be certain of their infection status (i.e. that died after
364 the detection threshold on day 15) vs. individuals that died before this threshold day.

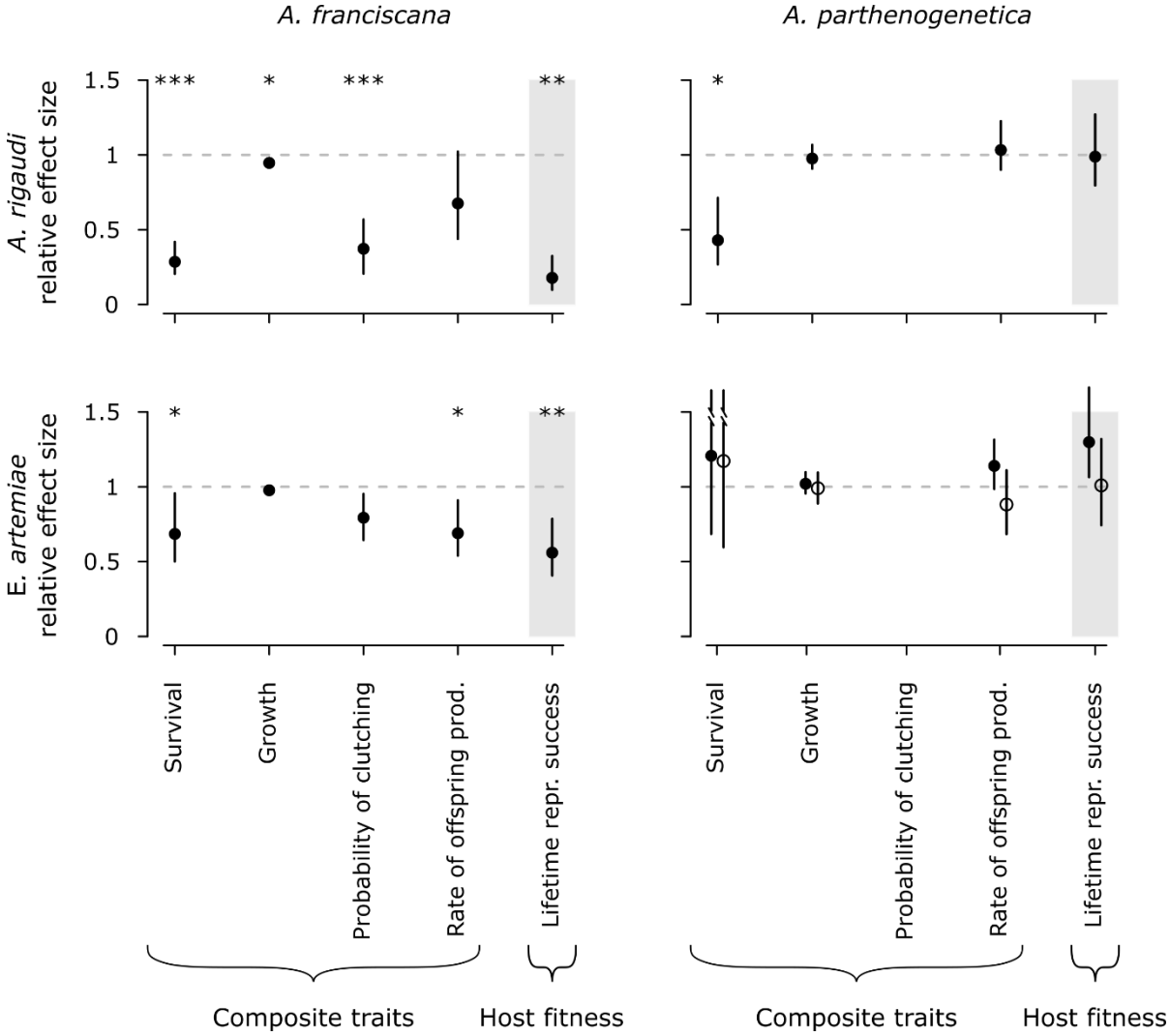
Host-parasite combination		Infection rate after vs. before the detection threshold	
A. franciscana			
Exposure to *A. rigaudi*		86 %	50 %
Exposure to *E. artemiae*		96 %	13 %
A. parthenogenetica			
Exposure to *A. rigaudi*		100 %	15 %
Exposure to *E. artemiae*	– low spore dose	64 %	0 %
	– high spore dose	86 %	20 %

365

366 *Species-level effects – Virulence of infections*

367 We analyzed the species-level results of Experiment 2 in two parts. First, we analyzed the virulence
368 of parasite infections, expressed as effects on host survival, growth, and reproduction. These results
369 are summarized in Fig. 2 and the significance of tested effects is listed in Table 4; we discuss the
370 effects of infection in more detail below. Here, we report only the analyses for infected vs. control
371 individuals, which excluded all individuals that died before day 15. When we compared exposed vs.
372 control individuals that died before the cut-off day the results were not qualitatively different.

373 **Figure 2. Host fitness (≈ parasite virulence) in the four host-parasite combinations.** All factors are shown as
374 fitted effects relative to controls: survival is an acceleration factor (the ratio of expected time-until-death); the
375 probability of reproduction is a relative risk; growth, rate of offspring production, and LRS are ratios. Bars
376 represent the 95% profile likelihood CIs (survival) or bootstrapped CIs (all others). *A. parthenogenetica*
377 infected after exposure to 10 000 *E. artemiae* spores are indicated with open circles. The reference (controls) is
378 indicated by the dotted gray line; asterisks indicate significant differences from controls.
379 The plotted survival effect for *A. parthenogenetica* excludes the aberrant group (Batch 34 ± 2 days old, Size
380 class 7.5 mm, see Results). All reproductive and fitness traits were obtained for females only. The probability
381 of reproduction is not shown for *A. parthenogenetica* because it could not be analyzed. Weighing the
382 contributions of nauplii and cysts to the rate of offspring production and LRS generated qualitatively
383 equivalent results; the results shown here are for equal weights.



384

385 **Table 4. Significance of tested effects for the virulence of infections.** Analyses were run separately for *A.*
386 *franciscana* and *A. parthenogenetica*. See text for post-hoc analyses of Treatment.

Tested variable	Fixed-effect terms	test statistic, *p*	Effect
Virulence of infections: *A. franciscana*			
Survival	Treatment	$\chi^2(2) = 48.2$, $p < 0.0001$	↓ when infected
	Sex	$\chi^2(1) = 33.2$, $p < 0.0001$	↑ for males
	Size class	$\chi^2(1) = 4.3$, $p = 0.04$	↑ for larger individuals
	interactions	all non-significant	
Growth between days 1 & 30	Treatment	$\chi^2(2) = 9.7$, $p < 0.01$	
	Sex	$\chi^2(1) = 133.5$, $p < 0.0001$	↓ for males
	Size class	$\chi^2(1) = 95.0$, $p < 0.0001$	↓ for larger individuals
	interactions	all non-significant	
Reproduction			
Time until sexual maturity	Treatment	$\chi^2(2) = 22.5$, $p < 0.0001$	↑ when infected
	Size class	$\chi^2(1) = 0.1$, $p = 0.83$	
	interaction	$\chi^2(2) = 1.3$, $p = 0.54$	
Probability of producing a clutch	Treatment	$\chi^2(2) = 31.3$, $p < 0.0001$	↓ when infected
	Size class	$\chi^2(1) = 0.5$, $p = 0.50$	
	interaction	$\chi^2(2) = 0.8$, $p = 0.69$	
Rate of offspring production*	Treatment	$\chi^2(2) \geq 7.9$, $p \leq 0.02$	↓ when infected
	Size class	$\chi^2(1) \leq 0.5$, $p \geq 0.50$	
	interaction	$\chi^2(2) \leq 1.8$, $p \geq 0.41$	
Timing of offspring production*	Treatm. : % Repr. period	$\chi^2(4) \leq 3.7$, $p \geq 0.45$	
Type of offspring produced	Treatment	$\chi^2(2) = 16.8$, $p < 0.001$	more nauplii when infected
	Size class	$\chi^2(1) = 0.1$, $p = 0.73$	
	interaction	$\chi^2(2) = 0.7$, $p = 0.71$	
Fitness (LRS*)	Treatment	$\chi^2(4) \geq 46.6$, $p < 0.0001$	↓ when infected
	Size class	$\chi^2(2) \leq 1.5$, $p \geq 0.48$	
	Interaction	$\chi^2(4) \leq 1.5$, $p \geq 0.82$	
Virulence of infections: *A. parthenogenetica*			
Survival	Treatment	$\chi^2(3) = 19.7$, $p < 0.001$	↓ when infected
	Size class	$\chi^2(2) = 11.5$, $p < 0.01$	↑ for larger individuals
	interaction	see text	
Growth between days 1 & 30	Treatment	$\chi^2(3) = 1.3$, $p = 0.73$	
	Size class	$\chi^2(2) = 35.8$, $p < 0.0001$	↓ for larger individuals
	interaction	$\chi^2(6) = 13.1$, $p = 0.04$	see text
Reproduction			
Rate of offspring production*	Treatment	$\chi^2(3) \leq 5.8$, $p > 0.12$	
	Size class	$\chi^2(2) \geq 8.8$, $p = 0.01$	↑ for larger individuals
	interaction	$\chi^2(6) \leq 10.4$, $p \geq 0.11$	
Timing of offspring production*	Treatm. : % Repr. period	$\chi^2(4) \geq 10.4$, $p < 0.11$	earlier when infected
Type of offspring produced*	Treatment	$\chi^2(2) = 1.4$, $p = 0.71$	
	Size class	$\chi^2(2) = 0.1$, $p = 0.96$	
	interaction	$\chi^2(6) = 9.1$, $p = 0.17$	
Fitness (LRS*)	Treatment	$\chi^2(6) \leq 5.6$, $p \geq 0.13$	
	Size class	$\chi^2(4) \geq 8.2$, $p < 0.09$	↓ for largest individuals
	interaction	$\chi^2(12) \leq 13.8$, $p \geq 0.31$	

387 *Depending on the weight of nauplii vs. cysts.

388 In most host-parasite combinations, survival was reduced (Fig. 2, Table 4). For *A. franciscana*, a
389 lognormal survival model best fit the data ($\Delta AICc \geq 4.2$). Infection significantly reduced survival; post-
390 hoc testing revealed that this effect was highly significant for *A. rigaudi* and marginally significant for
391 *E. artemiae* ($t = -6.7$ and -2.2, $p < 0.0001$ and $p = 0.05$, respectively). For *A. parthenogenetica*, a log-
392 logistic survival model best fit the data ($\Delta AICc \geq 0.9$). Survival was affected by infection, size class,
393 and their interaction, but this complicated interaction effect was due to the aberrant survival curves

of one group of individuals (Batch 34 ± 2 days old, Size class 7.5 mm), which had high death rates for controls and low death rates for infected hosts. When this group was removed, the interaction effect became non-significant. In general therefore, survival of *A. parthenogenetica* was reduced by infection with a parasite; post-hoc testing revealed that individuals infected with *A. rigaudi* had significantly lower survival ($t = -3.3$, $p < 0.01$), while individuals infected with *E. artemiae* did not ($t = 0.7$ and 0.5, $p = 0.86$ and 0.94 for low and high spore dose, respectively).

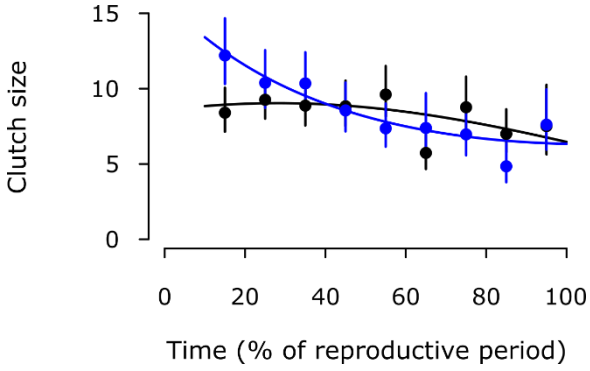
More than 90% of host growth occurred between days 1 and 30 (Supp. Table 2), so only this period was analyzed (Fig. 2, Table 4). For *A. franciscana*, infection significantly reduced growth; this effect was driven by *A. rigaudi* (post-hoc $z = -3.1$, $p < 0.01$) and non-significant for *E. artemiae* (post-hoc $z = -1.5$, $p = 0.23$). *A. parthenogenetica* growth was affected by infection interacting with size class, with the smallest size class growing slightly less when infected with a high dose of *E. artemiae*, and the middle size class growing slightly more when infected with either dose of *E. artemiae*.

Parasite infection affected the reproduction of *A. franciscana* females in various ways (Fig. 2, Table 4). The time until maturity, which was best described by a lognormal distribution ($\Delta AICc \geq 4.7$), was significantly delayed by infection with either parasite species (post-hoc for *A. rigaudi* $t = 4.6$, $p < 0.0001$; post-hoc for *E. artemiae* $t = 2.5$, $p = 0.02$). The probability that *A. franciscana* females produced a clutch was also significantly lower when they were infected; this effect was driven by *A. rigaudi* (post-hoc $z = -5.3$, $p < 0.0001$) and was marginally non-significant for *E. artemiae* (post-hoc $z = -2.1$, $p = 0.06$). For *A. franciscana* females that did reproduce, infection with *A. rigaudi* increased the proportion of nauplii clutches (post-hoc for *A. rigaudi* $z = 3.9$, $p < 0.001$; post-hoc for *E. artemiae* $z = 0.8$, $p = 0.68$). The rate of offspring production was significantly reduced by infection with *E. artemiae* for all weights of nauplii vs. cysts (post-hoc for *E. artemiae* $z \leq -2.8$, $p \leq 0.01$), but was only significantly reduced by infection with *A. rigaudi* when cysts were weighted twice as much as nauplii ($z = -2.3$, $p = 0.04$). Finally, the timing of offspring production was independent of infection status.

In contrast, parasite infection had little effect on the reproduction of *A. parthenogenetica* females (Fig. 2, Table 4). The effects of infection on the time until sexual maturity or the probability of producing a clutch could not be tested, because almost all *A. parthenogenetica* females started reproducing immediately. For reproducing females, neither the proportion of live clutches, nor the rate of offspring production were affected by infection with either parasite. However, infection with *A. rigaudi* did lead to a significant shift towards earlier reproduction (Fig. 3; significant when cysts were weighted equally or doubly compared to nauplii; post-hoc for *A. rigaudi* $z \geq 2.6$, $p \leq 0.03$ for all weights of nauplii vs. cysts; post-hoc for *E. artemiae* $z \leq 1.1$, $p \geq 0.58$ for all weights of nauplii vs. cysts).

Figure 3. Timing of reproduction in *A. parthenogenetica* controls (black) and infected with *A. rigaudi* (blue). Lines represent the prediction of the best model, points and vertical bars give the observed means and their

95% CIs, calculated over intervals of 10%. Weighing the contributions of nauplii and cysts to the total number of offspring generated qualitatively similar results; the results shown here are for equal weights.



The fitness of female hosts – estimated by the lifetime reproductive success (LRS), i.e. the total number of offspring produced – was significantly reduced by infection with either parasite for *A. franciscana*, but not for *A. parthenogenetica* (Fig. 2, Table 4).

Species-level effects – Parasite transmission and fitness

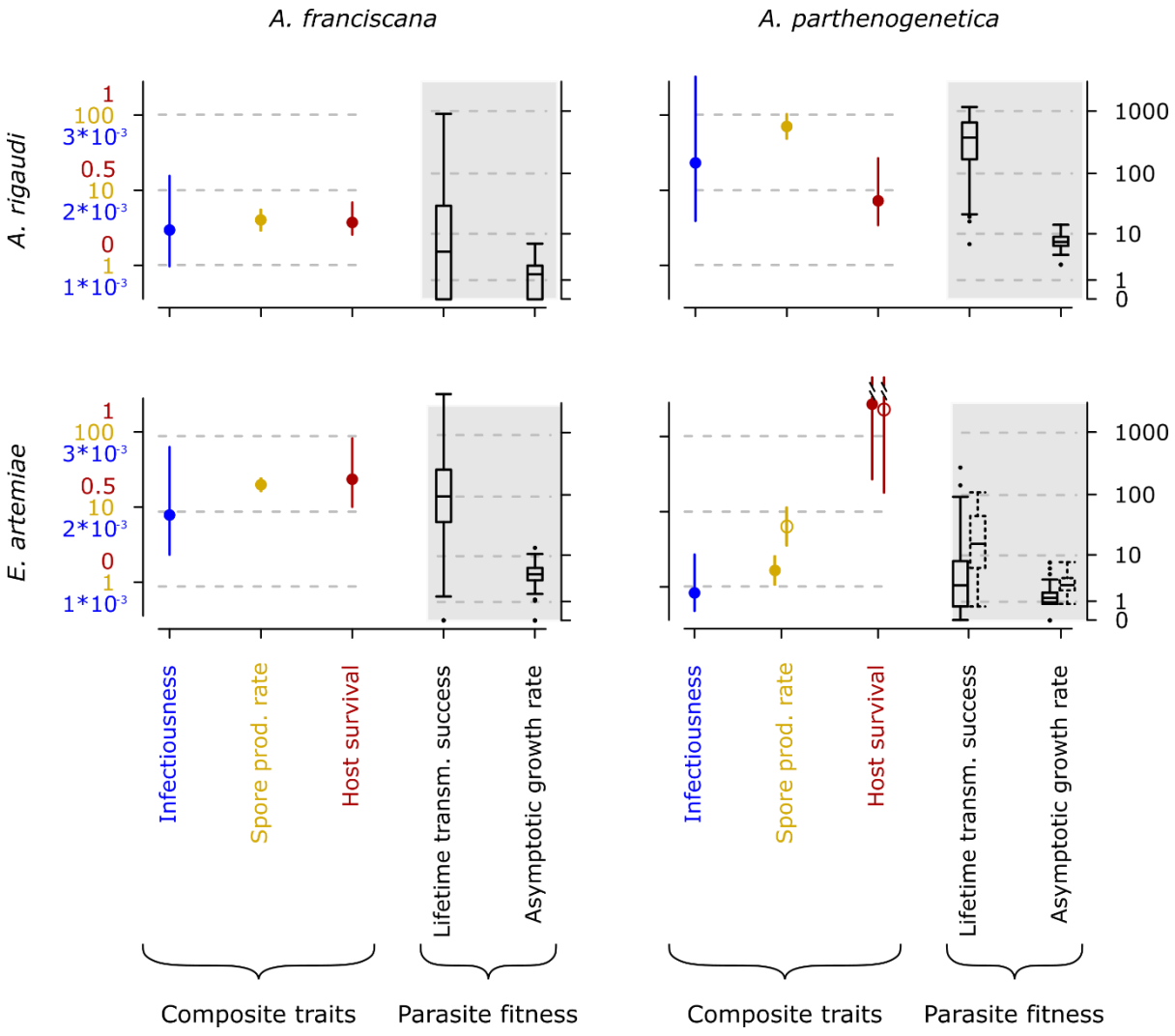
Second, we studied the effects of the host species on the parasite's transmission and fitness (summarized in Fig. 4). These analyses were combined for all host-parasite combinations, but *A. parthenogenetica* that were exposed to 10 000 *E. artemiae* spores were analyzed separately unless otherwise specified.

The infectiousness of a single spore (the probability that it started a detectable infection, as calculated using the transmission data) corresponded with our expectations based on Experiment 1 (Fig. 4). Host-parasite combination had a significant effect on infectiousness ($\chi^2(1) = 16.7$, $p < 0.0001$). *A. rigaudi* tended to be more infectious to *A. parthenogenetica* than to *A. franciscana* (post-hoc $z = 2.3$, $p = 0.10$); *E. artemiae* was significantly more infectious to *A. franciscana* than to *A. parthenogenetica* (post-hoc $z = 3.6$, $p < 0.01$).

The rates of spore production were significantly different in all host-parasite combinations (overall $\chi^2(1) = 205.9$, $p < 0.0001$; all post-hoc pairwise comparisons $z \geq 3.2$, $p < 0.01$; Fig. 4); they were highest in the combinations *A. parthenogenetica-A. rigaudi* and *A. franciscana-E. artemiae*. For *A. parthenogenetica* infected with *E. artemiae*, the rate of spore production was notably higher when the initial inoculum was larger ($\chi^2(1) = 10.6$, $p = 0.001$). Within the overall differences in spore production rate between host-parasite combinations, spore production also varied over time: rates peaked on day 30 in the combinations *A. parthenogenetica-A. rigaudi* and *A. franciscana-E. artemiae*, and increased throughout the experiment in the other combinations (results not shown). These results will be discussed in more detail in a follow-up paper (Lievens et al. *in prep*).

As expected, transmission success increased with the rate of spore production in all host-parasite combinations (Spearman's ρ between 0.57 and 0.69, $p < 0.0001$; Supp. Fig. 1).

457 **Figure 4. Parasite fitness in the four host-parasite combinations.** The composite traits infectiousness
458 (probability of infection by a single spore), rate of spore production (# counted spores/5 days, *ln* scale), and
459 host survival (which determines infection duration, copied from Fig. 2) are shown as fitted means with 95%
460 profile likelihood CIs. The fitness measures lifetime transmission success (*ln + 1* scale) and asymptotic growth
461 rate (*ln + 1* scale) are shown as Tukey box plots. *A. parthenogenetica* infected after exposure to 10 000 *E.*
462 *artemiae* spores are indicated with open circles and dotted box plots.



463

464 Because the number of spores produced correlated with transmission success, we were able to use
465 the lifetime transmission success and asymptotic growth rate as indicators of parasite fitness. The
466 two measures were tightly correlated (Supp. Fig. 2) and both differed across host-parasite
467 combinations ($\chi^2(4) = 189.9$ and 245.0, respectively, $p < 0.0001$; Fig. 4). The fitness of *A. rigaudi* was
468 highest in *A. parthenogenetica*; that of *E. artemiae* was highest in *A. franciscana.* All host-parasite
469 combinations were significantly different except the low-performers *A. parthenogenetica-E.*
470 *artemiae* and *A. franciscana-A. rigaudi* (post-hoc $p < 0.001$ vs. $p = 0.46$ and $p \leq 0.04$ vs. $p = 0.69$,
471 respectively). This was true for both spore doses of *A. parthenogenetica-E. artemiae* regarding the
472 lifetime transmission success, but only for the low spore dose for the asymptotic growth rate.

473 *Individual variation in resistance*

474 Among the individuals that survived until we could be certain of their infection status, the rate of
475 resistance varied between 0 and 36% in the different host-parasite combinations (Table 5). For *A.*

476 *franciscana*, significantly more individuals resisted infection with *A. rigaudi* than with *E. artemiae*
477 ($\chi^2(1) = 10.4$, $p < 0.01$), and this effect was independent of sex ($\chi^2(1) = 0.3$, $p = 0.58$). Similarly,
478 significantly more *A. parthenogenetica* resisted infection with *E. artemiae* ($\chi^2(1) = 20.6$, $p < 0.0001$),
479 with a marginally non-significant difference between the two spore doses ($\chi^2(1) = 3.8$, $p = 0.052$).
480 There was substantial variation in infection outcome for the combinations *A. franciscana*/'Exposure
481 to *A. rigaudi*', and *A. parthenogenetica*/'Exposure to *E. artemiae* – low dose', so we continued our
482 analyses with these combinations.

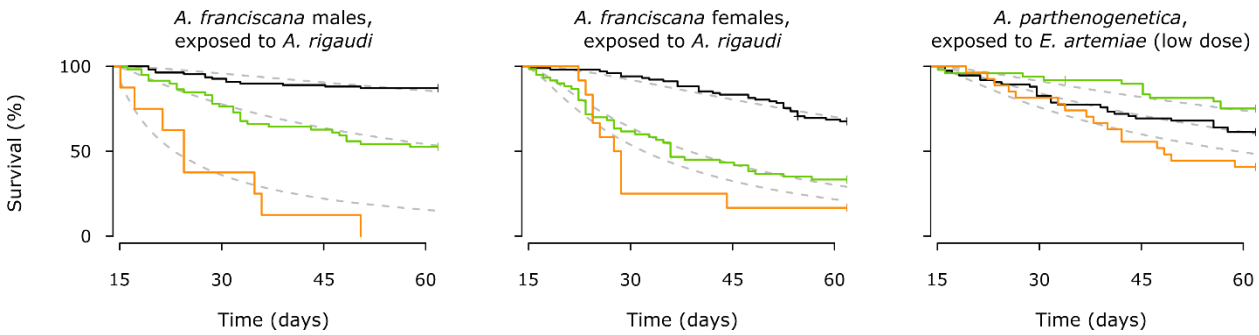
483 **Table 5. Number of exposed individuals that were infected or resistant to *A. rigaudi* and *E. artemiae*.** These
484 counts excluded all individuals who died before infection status could be definitively determined, i.e. those
485 that died before day 15 of the experiment.

Host-parasite combination	Resistant	Infected	% Resistant
***A. franciscana* males**			
Exposure to *A. rigaudi*	8	59	12%
Exposure to *E. artemiae*	5	106	5%
***A. franciscana* females**			
Exposure to *A. rigaudi*	12	60	17%
Exposure to *E. artemiae*	5	114	4%
A. parthenogenetica			
Exposure to *A. rigaudi*	0	62	0%
Exposure to *E. artemiae* – low spore dose	27	49	36%
– high spore dose	4	25	14%

486

487 For both *A. franciscana* exposed to *A. rigaudi* and *A. parthenogenetica* exposed to a low spore dose
488 of *E. artemiae*, resistant individuals died more quickly than infected individuals (Fig. 5; ΔAICc
489 respectively > 4.4 and = 1.6, Supp. Table 3). For *A. franciscana*, resistant males had a higher mortality
490 than resistant females (Fig. 5; ΔAICc > 1.7, Supp. Table 3).

491 **Figure 5. Survival curves for resistant (orange), infected (green), and control (black) individuals.** Note that
492 these curves start at day 15, i.e. when infection status could be fully ascertained. The curves shown here are
493 averaged across size class and origin for *A. franciscana* and across size classes in *A. parthenogenetica*. Model
494 estimates for each curve are plotted in gray.



495

496 Finally, there was little support for an effect of resistance on reproduction in females of either host
497 species (Supp. Table 4). *A. franciscana* females that resisted infection with *A. rigaudi* behaved
498 similarly to females that became infected (strong effects of *Resistant-Infected-Control*, but no or
499 weak support for a difference between resistant and infected females). The reproductive behavior of
500 *A. parthenogenetica* females that resisted infection with a low dose of *E. artemiae* was similar to
501 that of infected and control females.

502 ## DISCUSSION

503 The degree of host specialization is a key property of any multi-host parasite. Host specialization,
504 when considered as a difference in fitness, arises from a series of life history traits including the
505 ability to infect, virulence, and the rate of transmission. We quantified these traits for two
506 microsporidian gut parasites (*A. rigaudi* and *E. artemiae*) infecting two brine shrimp hosts (*A.*
507 *franciscana* and *A. parthenogenetica*), by tracking the life history of both hosts and parasites after
508 experimental infection. A brief synopsis of the results is shown in Table 6.

509 Overall, each of the parasites was partially specialized: *A. rigaudi* was very successful in *A.*
510 *parthenogenetica*, while *E. artemiae* performed best in *A. franciscana*. Below, we discuss how the
511 individual life-history traits combine to shape the degree of specialization, and the ensuing effects of
512 specialization on the hosts. We refer to the host-parasite combinations where parasites reached
513 high fitness as the 'matched' combinations (Table 6). The reversed combinations also produced
514 viable transmission stages, but at much lower rates; we will call these the 'mismatched'
515 combinations.

516 **Table 6.** Qualitative synopsis of results.

	Parasite species	
Host species	***A. rigaudi***	***E. artemiae***
A. franciscana	moderately infectious highly virulent low spore production ⇨ **low parasite fitness, mismatched host & parasite**	highly infectious moderately virulent high spore production ⇨ **high parasite fitness, matched host & parasite**
A. parthenogenetica	highly infectious moderately virulent (survival only) high spore production ⇨ **high parasite fitness, matched host & parasite**	poorly infectious avirulent low spore production ⇨ **low parasite fitness, mismatched host & parasite**

517

518 ### *Partial specialization via a mix of specialist and generalist traits*

519 Specialization is often presented as a dichotomy: specialists, whose fitness is high or null for
520 different hosts, versus generalists, who generally have intermediate fitness on several hosts (Leggett
521 et al., 2013; Poulin, 2007, chap. 3; Schmid-Hempel, 2011, chap. 7). *A. rigaudi* and *E. artemiae* fall into
522 a gray zone between these categories, being neither absolute specialists – they can exploit both
523 hosts –, nor absolute generalists – their fitness is much higher in the matched hosts.

524 When broken down into its composite traits, the origin of this partial specialization becomes clear.
525 Parasites should be as infective as possible to hosts to which they are adapted, and indeed both *A.*
526 *rigaudi* and *E. artemiae* are highly infectious to their matched hosts. Similarly, we expect strong
527 transmission to be advantageous, and accordingly we find that both parasites have high rates of
528 spore production in their matched hosts. The expectations for virulence are not as clear-cut. A
529 'Darwinian devil' parasite would be avirulent while maintaining high transmission rates, but it is
530 generally considered that these two factors are correlated (Alizon et al., 2009). Virulence must
531 therefore be judged in relation to transmission; for example, high virulence can be adaptive if
532 coupled with high rates of transmission, or maladaptive if not. When considered in this way, *A.*
533 *rigaudi* and *E. artemiae*'s virulence are also coherent with their overall specialization. *A. rigaudi*
534 causes high survival virulence – and thus short infection durations – in both hosts, but this is

advantageously coupled with high rates of spore production in its matched host *A. parthenogenetica*, and disadvantageously coupled with low rates of spore production in its mismatched host. *E. artemiae* is avirulent in its mismatched host, which at first glance appears ideal. However, when spore production is taken into account, it becomes clear that this avirulence in *A. parthenogenetica* is coupled with very low rates of transmission, whereas the rate of spore production is high in *A. franciscana*.

Despite this, were we to consider the composite traits individually, they would not all lead us to conclude that the two parasites are partially specialized. The pattern of spore production in the matched vs. mismatched combinations best reflects the overall degree of specialization. Infectivity, on its own, might lead us to conclude that *E. artemiae* is a specialist while *A. rigaudi* is more generalist (also discussed in Lievens et al., n.d.). Virulence is difficult to interpret outside the context of spore production, as discussed above, making it a particularly poor proxy for overall specialization. Integrating across all of these life history traits is therefore necessary to properly understand the nature of this host-parasite system, and will probably have important implications for the evolution of virulence (Alizon and Michalakis, 2015) and infection success (Hall et al., 2017).

Mismatched parasites have different kinds of suboptimal virulence

Several theoretical predictions have been made for the evolution of virulence in multi-host parasites that are specialized on one host and spill over into another (source-sink dynamics), all of which agree that virulence should depend exclusively on the optimum in the specialized host (Dobson, 2004; Gandon, 2004; Regoes et al., 2000; Woolhouse et al., 2001). Predictions of virulence in the non-specialized host, however, vary. Regoes et al. considered virulence to be coupled to exploitation, which trades off between hosts; their prediction is that the parasite will be avirulent in the spillover host. Gandon also considered virulence to be coupled to exploitation, but in his model the level of exploitation is correlated between hosts. In this case, the parasite can be maladaptively avirulent or hypervirulent in the spillover host, depending on the relative resistances of the hosts. Finally, Woolhouse et al. pointed out that virulence can become decoupled from parasite exploitation in spillover hosts, for example through harmful immune responses (Graham et al., 2005), leading to maladaptively high virulence (see also Leggett et al., 2013). Empirically, virulence patterns across multiple hosts have only rarely been studied in natural systems (Rigaud et al., 2010), so it is difficult to determine which of these possibilities may be more common.

In the mismatched hosts of our *Artemia*-microsporidian system, two different virulence patterns are apparent. First, in the combination *A. franciscana*-*A. rigaudi*, the parasite is very virulent on a host in which it can barely reproduce. Its virulence in the non-specialized host is thus decoupled from exploitation and maladaptive, matching Woolhouse et al. (2001)'s prediction for unconstrainedly high virulence. The situation of *A. rigaudi* strongly resembles that of the generalist microsporidian parasite *Nosema bombi*, which infects bumble bees. Two of *N. bombi*'s most important hosts are *Bombus terrestris*, in whom it is so virulent that it cripples its own year-to-year transmission, and *Bombus lucorum*, in whom its virulence is moderate enough to allow transmission (Rutrecht and Brown, 2009). A number of zoonotic human diseases also fit this pattern (Woolhouse et al., 2001). In contrast, in the mismatched combination *A. parthenogenetica*-*E. artemiae*, the parasite is avirulent. *E. artemiae* could therefore correspond to the situations described by Regoes et al. (2000) and Gandon (2004), in which a non-specialized host is under-exploited and suffers no virulence. Indeed, *A. parthenogenetica* is also less susceptible to *E. artemiae*, giving some support to Gandon's scenario of differently resistant hosts. A similar case could be made for the nematode *Howardula aoronymphium* (Jaenike and Dombeck, 1998; Jaenike, 1996; Perlman and Jaenike, 2003) and for the

580 Drosophila C virus (Longdon et al., 2015), which exhibit a range of exploitation and correlated
581 virulence across host species.

582 Overall, our results provide support for the varied possible theoretical predictions of virulence
583 evolution in multi-host parasites: in one case, we appear to be dealing with decoupled, 'runaway'
584 virulence, while in the second the differences in virulence may be driven by levels of host resistance.
585 These contrasting findings show that the different theoretical outcomes can even be found among
586 host-parasite pairs that are ecologically extremely similar and phylogenetically close.

587 *Mismatched hosts incur high costs of resistance*

588 In the matched host-parasite combinations, uninfected individuals were rare or nonexistent (Table
589 5), and suffered no detectable survival cost (data not shown). It is possible that an extremely high
590 mortality rate of resistant individuals caused them to die before we could reliably detect infection,
591 leading us to underestimate both the frequency and the cost of resistance. However, survival rates
592 for the matched combinations were universally high in the infectivity experiment, which lasted one
593 week. Any mortality conferred by resistance would therefore have to be incurred precisely in the
594 second week of infection, which is unlikely. It is more probable that the high rates of infection reflect
595 selection on the parasite to evade or overcome resistance in its matched host (Hasu et al., 2009).

596 In the mismatched host-parasite combinations, however, up to one third of the exposed hosts were
597 uninfected, and the life histories of these individuals differed clearly from those of control or
598 infected hosts (Table 5, Fig. 5). This suggests that their lack of infection was the result of an active
599 resistance mechanism. Because the parasite was absent, the effects of deploying resistance must
600 have been induced by the host itself, as a consequence of its immune reaction upon exposure
601 (Graham et al., 2005; immunopathology, Schmid-Hempel, 2003).

602 This resistance was extremely costly: resistant individuals died much more rapidly than control and
603 infected hosts (Fig. 5). Since there was no detectable compensation through increased fecundity, we
604 must conclude that resistance in these cases is maladaptive. This is intriguing, because *A. franciscana*
605 and *A. parthenogenetica* are regularly exposed to their mismatched parasites in the field (Lievens et
606 al., n.d.). Host resistance has been shown to evolve quickly in a similar host-parasite system
607 (*Daphnia magna-Octosporea bayeri*, Zbinden et al. 2008), so we would not expect maladaptive
608 resistance responses to persist in the host populations. An explanation may be that source-sink
609 dynamics acting in the parasite populations prevent them from evolving to reduce their impact on
610 the mismatched hosts. In turn, selection on the host to reduce its response to the mismatched
611 parasite could perhaps be countered by other factors, such as the need to maintain its overall
612 immune capacity (Graham et al., 2005). Similarly disproportionate costs of resistance, with
613 uninfected hosts dying more rapidly than even infected hosts, have been found in e.g. *Daphnia*
614 resisting the bacterium *Pasteuria* (though see Labbé et al., 2010; Little and Killick, 2007), and naïve
615 isopods resisting infection with a helminth (Hasu et al., 2009).

616 *Infection with* A. rigaudi *causes shifts in reproductive strategy*

617 *A. parthenogenetica* females infected with their matched parasite *A. rigaudi* died more quickly than
618 controls and did not produce offspring at a higher overall rate, yet did not suffer from reduced
619 lifetime reproductive success. They managed this by shifting towards earlier reproduction to
620 alleviate the survival virulence, a plastic behavior known as fecundity compensation (Agnew et al.,
621 2000; Chadwick and Little, 2005; cf. Minchella and Loverde, 1981) (Fig. 3). Further investigation
622 revealed that females accomplished this shift in reproductive effort by increasing the size, rather
623 than the frequency, of early clutches (data not shown). This is a new finding for *Artemia*, which

624 opens a number of interesting new avenues of research. For example, Mediterranean *A.*
625 *parthenogenetica* are also heavily infected with the castrating cestode *Flamingolepis liguloides*
626 (Amat et al., 1991). If fecundity compensation is also possible in the face of *F. liguloides* infections,
627 this would drastically change our understanding of the overall impact of this parasite. Alternately, a
628 recent study has shown that the fecundity compensation response of snails can be suppressed when
629 they undergo additional stress (Gleichsner et al., 2016). Studying the relationship between common
630 environmental stressors of *A. parthenogenetica*, such as salinity, and their ability to shift their
631 reproductive effort may help us understand the evolutionary relevance of such mechanisms.

632 *A. franciscana* females did not have a similar fecundity compensation response when infected with
633 either parasite. However, infections of *A. franciscana* with *A. rigaudi* were associated with an
634 interesting change in reproductive strategy. Infected females were less likely to produce a clutch,
635 but those that did reproduce were more likely to produce clutches of live young. Considering that
636 *Artemia* generally produce cysts when stressed (Clegg and Trotman, 2002), this result seems
637 counterintuitive. Perhaps *A. rigaudi* interferes with the cyst production mechanism, either
638 collaterally or as a manipulation to increase the availability of susceptible hosts. Another possibility
639 is that a shift towards live born offspring is advantageous for the host. If infected mothers can
640 produce offspring which are protected against the parasite, for example via transgenerational
641 immune priming (which Artemia can do, Norouzitallab et al., 2015), it may be more worthwhile to
642 produce protected nauplii over protected cysts: protected nauplii could have an immediate
643 competitive advantage in the parasite-infested environment, while the hatching environment of
644 protected cysts is unknown.

645 *Comparison with the field: previous & future results*

646 The results of this study are consistent with field observations of the *Artemia*-microsporidian system.
647 Our identification of the matched and mismatched host-parasite combinations is consistent with the
648 field data, which shows that *A. rigaudi* is dependent on its matched host to persist in the natural
649 host community, and suggests that the same may be true for *E. artemiae* (Lievens et al., n.d.). As *E.*
650 *artemiae* and *A. rigaudi* performed equally poorly in their mismatched hosts, this experiment
651 supports that suggestion. Our results for infectivity also reflect the consistently higher prevalence of
652 *A. rigaudi* and *E. artemiae* in respectively *A. parthenogenetica* and *A. franciscana* (Lievens et al., n.d.;
653 Rode et al., 2013). In addition, we find that *A. rigaudi* is considerably more virulent than *E. artemiae*
654 in both host species. Rode et al. (2013) reached a similar conclusion based on the reproductive state
655 of females collected from the field. Interestingly, the effect found by Rode et al. was that sexually
656 mature females of both species were less likely to be brooding a clutch when they were infected
657 with *A. rigaudi*, while in our study *A. rigaudi* did not affect the frequency of clutching once sexual
658 maturity had been reached (data not shown). The different conditions in the field may be
659 responsible for this seemingly additional virulence (e.g. food limitation, Brown et al. 2000,
660 Bedhomme et al. 2004, Vale et al. 2011; temperature, Mitchell et al. 2005, Vale et al. 2008).

661 Further insights into the relationship between the microsporidians and their *Artemia* hosts could
662 come from experimental coinfections. So far, we have examined the effects of *A. rigaudi* and *E.*
663 *artemiae* in isolation, but coinfections are very common in the field (Lievens et al., n.d.). Coinfection
664 often has profound effects on the expression of parasite virulence and the success of their
665 transmission, and can thus be expected to affect the evolution of microsporidian life history and
666 host responses (Alizon et al., 2013; Rigaud et al., 2010). Studying the effects of single vs. mixed
667 infections could therefore provide new perspectives into selection on ecological specialization in the
668 field.

Conclusion

In nature, multi-host parasites and multi-parasite hosts are likely to be the rule, rather than the exception (Cleaveland et al., 2001; Streicker et al., 2013; Taylor et al., 2001). Despite important research efforts in this direction, we still know little about the interplay between parasite specialization and its composite traits in these complex systems (Hall et al., 2017; Rigaud et al., 2010; Rutrecht and Brown, 2009). In this study, we quantified parasite infectivity, virulence, and spore production in all combinations of a naturally occurring two-host, two-parasite system. We showed that while both parasites can complete their life cycle on both hosts, each performs better on one of the two host species, and we therefore conclude that they are partially specialized. Studying the underlying life-history traits revealed the origin of this partial specialization: spore production was universally important while infectivity was more loosely related to overall specialization, and the correct 'tuning' of parasite virulence had a major impact on infections in non-specialized hosts. There were also consequences of specialization on the host side: one host species was able to alleviate the survival virulence of its specialized parasite through fecundity compensation, while non-specialized hosts suffered large costs of resistance. This diversity of strategies and outcomes occurred despite the ecological and phylogenetic similarity of the hosts and parasites, highlighting the diverse strategies with which parasites overcome (or not) the difficulty of adapting to multiple host species.

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858

859 ## SUPPLEMENTARY MATERIAL

860 **Supplementary Table 1.** Host survival during the infectivity experiment.

Parasite species	Dose (spores/individual)	Nb. exposed	Nb. died	% Survived
A. franciscana				
Controls	0	17	7	59%
A. rigaudi	400	20	3	85%
	800	20	3	85%
	1 600	20	1	95%
	3 200	20	6	70%
	6 400	20	4	80%
E. artemiae	400	20	10	50%
	800	20	4	80%
	1 600	20	9	55%
	3 200	20	1	95%
	6 400	20	8	60%
A. parthenogenetica				
Controls	0	4	0	100%
A. rigaudi	400	16	0	100%
	800	20	1	95%
	1 600	20	0	100%
	3 200	8	0	100%
	6 400	4	0	100%
E. artemiae	400	20	2	90%
	800	20	0	100%
	1 600	20	1	95%
	3 200	20	2	90%
	6 400	20	2	90%

861

862 **Supplementary Table 2.** Results of paired t-tests comparing host growth before and after day 30 (all
863 treatments combined).

Hosts	Mean difference	p (mean difference ≠ 0)
***A. franciscana* males**		
Growth between days 1 & 30	2.1	< 0.0001
Growth between days 30 & 60	0.0	0.87
***A. franciscana* females**		
Growth between days 1 & 30	2.8	< 0.0001
Growth between days 30 & 60	0.3	< 0.0001
A. parthenogenetica		
Growth between days 1 & 30	1.7	< 0.0001
Growth between days 30 & 60	0.3	< 0.0001

864

865

866 **Supplementary Table 3.** Model comparison: link between survival and infection success. For each host-
867 parasite combination, these models grouped all the individuals exposed to that parasite (possible outcomes:
868 uninfected or infected) and the control individuals for that host into the factor *Resistant-Infected-Control*
869 (abbreviated Res-Inf-Ctrl). *Resistant-Infected-Control* was allowed to interact with all of the experimentally
870 manipulated factors (the base model). The base model for *A. franciscana* included *Sex*Size class* and a frailty
871 component for *Origin* (lognormal distribution, see Results). The base model for *A. parthenogenetica* included
872 *Size class* and a frailty component for *Batch* (log-logistic distribution, see Results). We used contrast
873 manipulation to detect how resistant, infected and control individuals differed (only models within ΔAICc = 3 of
874 the best contrast-manipulated model are shown). Note that these analyses only included individuals that
875 survived until at least day 15, when infection status could be definitively determined. *w* is the Akaike weight of
876 each model.

Host-parasite combination		**AICc**	**ΔAICc**	***w***
A. franciscana* exposed to *A. rigaudi				
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Sex		1367.1	0	0.53
Contrast manipulation:	*Ctrl > Inf > Res*	*1367.1*		
Base model + Res-Inf-Ctrl		1368.8	1.7	0.23
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Sex + Res-Inf-Ctrl : Size class		1369.3	2.2	0.18
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		1371.5	4.4	0.06
Base model		1429.3	62.2	0.00
***A. parthenogenetica* exposed to *E. artemiae* - low spore dose**				
Base model + Res-Inf-Ctrl		641.5	0	0.64
Contrast manipulation:	*Inf > Ctrl = Res*	*640.5*		
	Inf > Ctrl > Res	*641.5*		
	Inf = Ctrl > Res	*642.3*		
Base model		643.1	1.6	0.29
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		646.0	4.5	0.07

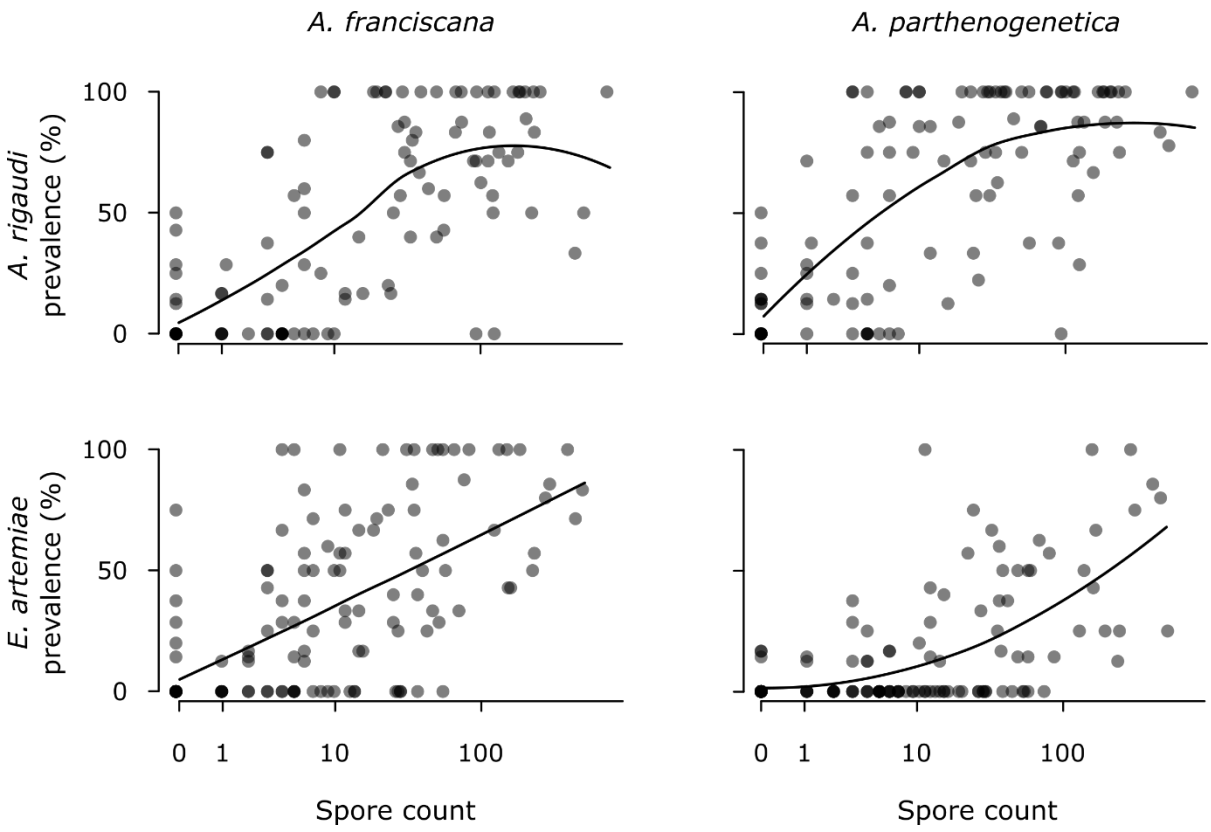
877

878 **Supplementary Table 4.** Model comparison: link between reproduction and infection success. For each host-parasite
879 combination, these models grouped all the individuals exposed to that parasite (possible outcomes: uninfected or
880 infected) and the control individuals for that host into the factor *Resistant-Infected-Control* (abbreviated Res-Inf-
881 Ctrl). *Resistant-Infected-Control* was allowed to interact with all of the experimentally manipulated factors (the base
882 model). The base model for *A. franciscana* included *Size class* and *Origin* as a random or frailty component; the base
883 model for *A. parthenogenetica* included *Size class* and *Batch* as a random effect. We used contrast manipulation to
884 detect how resistant, infected and control individuals differed (only models within ΔAICc = 3 of the best contrast-
885 manipulated model are shown). Note that these analyses only included individuals that survived until at least day 15,
886 when infection status could be definitively determined. *w* is the Akaike weight of each model.

Host-parasite combination		AICc	ΔAICc	*w*
***A. franciscana* exposed to *A. rigaudi*: time until sexual maturity**				
Base model + Res-Inf-Ctrl		951.6	0	0.82
Contrast manipulation:	*Inf = Res > Ctrl*	*949.7*		
	Inf > Res > Ctrl	*951.8*		
	Inf > Res = Inf	*953.2*		
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		954.6	3.0	0.18
Base model		974.4	22.8	0.00
***A. franciscana* exposed to *A. rigaudi*: probability of reproduction†**				
Base model + Res-Inf-Ctrl		205.5	0	0.75
Contrast manipulation:	*Ctrl > Inf = Res*	*204.0*		
	Ctrl > Inf > Res	*205.5*		
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		207.8	2.3	0.24
Base model		239.9	34.4	0.00
***A. franciscana* exposed to *A. rigaudi*: clutch type** (higher = more nauplii)**†**				
Base model + Res-Inf-Ctrl		306.7	0	1.00
Contrast manipulation:	*Res > Inf > Ctrl*	*306.7*		
	Res = Inf > Ctrl	*307.3*		
Base model		326.4	19.7	0.00
***A. franciscana* exposed to *A. rigaudi*: rate of offspring production*†**				
Base model + Res-Inf-Ctrl		96.0	0	0.66
Contrast manipulation:	*Res > Ctrl = Inf*	*95.4*		
	Res > Ctrl > Inf	*96.0*		
	Res = Ctrl > Inf	*98.2*		
Base model		98.3	2.3	0.21
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		99.3	3.3	0.13
***A. franciscana* exposed to *A. rigaudi*: Fitness (LRS)*†**				
Base model + Res-Inf-Ctrl		1094.0	0	1.00
Contrast manipulation:	*Ctrl > Res = Inf*	*1095.5*		
	Ctrl > Res > Inf	*1097.6*		
Base model		1145.7	51.7	0.00
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		-	-	-
***A. parthenogenetica* exposed to *E. artemiae* - low spore dose: clutch type** (higher = more nauplii)				
Base model + Res-Inf-Ctrl+ Res-Inf-Ctrl : Size class		315.6	0	0.76
Contrast manipulation:	*order of effects dependent on Size class*	*313.3*		
	order of effects dependent on Size class	*313.4*		
	order of effects dependent on Size class	*315.6*		
Base model + Res-Inf-Ctrl		318.3	2.7	0.20
Base model		321.2	5.6	0.05
A. parthenogenetica* exposed to *E. artemiae* - low spore dose: rate of offspring production				
Base model		181.1	0	0.96
Base model + Res-Inf-Ctrl		187.3	6.2	0.04
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		196.9	15.8	0.00
A. franciscana* exposed to *A. rigaudi*: Fitness (LRS)				
Base model + Res-Inf-Ctrl		1443.2	0	0.86
Contrast manipulation:	*Inf > Res = Ctrl*	*1441.4*		
	Inf = Res > Ctrl	*1441.8*		
	Inf > Res > Ctrl	*1443.2*		
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class		1446.8	3.6	0.14
Base model		1466.5	23.3	0.00

887 *Shown for the models that weighted nauplii and cysts equally; giving either offspring type a double weight produces
888 qualitatively equivalent results. †Only two resistant females reproduced, so these results should be interpreted with caution.

889 **Supplementary Figure 1. Spore production and transmission success in the four host-parasite combinations.**
890 These graphs relate the infection success (percentage of recipients infected) to the spore count in the
891 corresponding spore sample (*ln* + 1 scale) for *A. rigaudi* (top) and *E. artemiae* (bottom). Note that the graphs
892 are divided by recipient species, not donor species (see Methods). Each point represents a recipient group;
893 overlapping points shade to black. Lines represent 2nd-degree polynomial local regression (LOESS) fittings.



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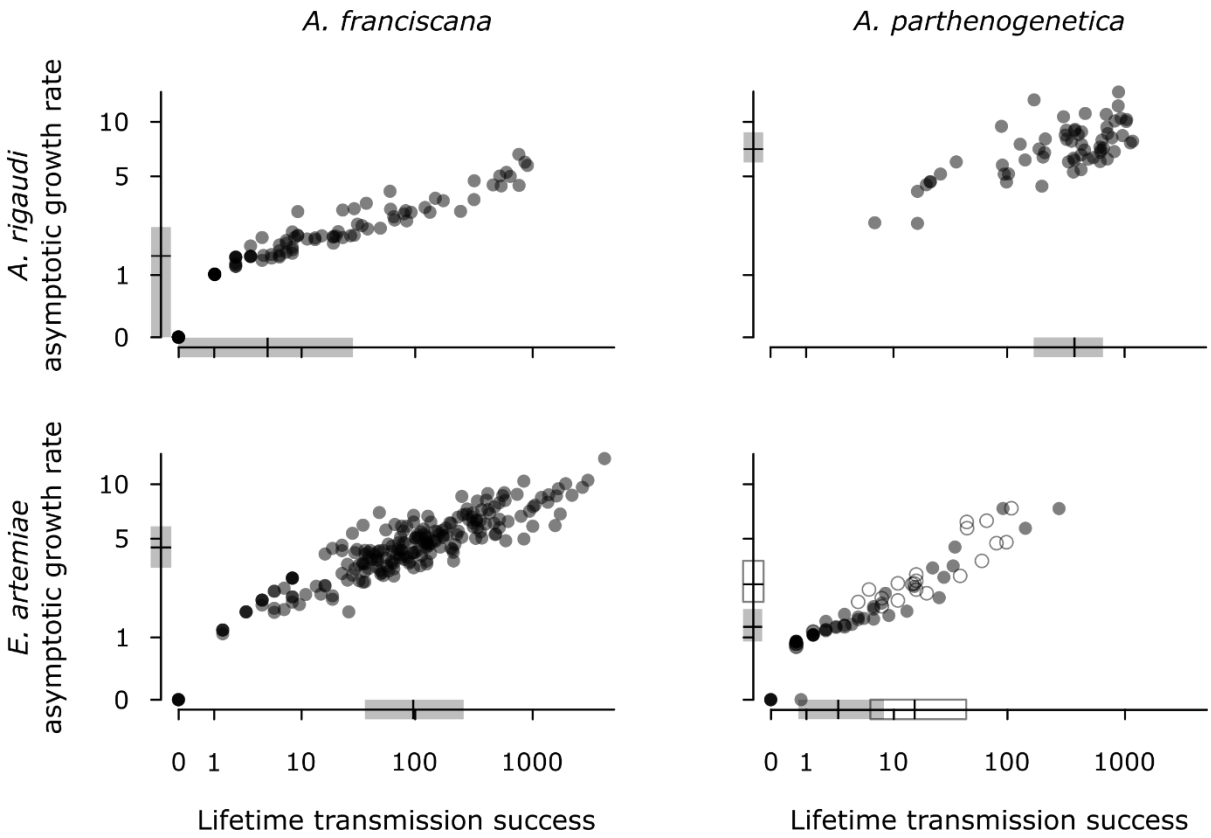
Supplementary Figure 2. Overall fitness measures of *A. rigaudi* (top) and *E. artemiae* (bottom) infections. The asymptotic growth rate (*ln* + 1 scale) is shown as a function of the lifetime transmission success (*ln* + 1 scale). The asymptotic growth rate should be maximized during epidemics, while the lifetime transmission success, as an estimator of R_0, should be maximized in endemic conditions. The median, first and third quartiles are shown by boxplots on the axes. For *A. parthenogenetica* infected with *E. artemiae*, the open circles and boxplot represent the females exposed to a high spore dose. Each point represents an infected host; overlapping points shade to black.