



Des défis biotiques pour des extrêmophiles : l'interférence reproductive et la spécialisation parasitaire chez Artemia

Eva J.P. Lievens

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THÈSE

Pour obtenir le grade de
Docteur

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Présentée par **Eva J. P. Lievens**

**Biotic challenges for extremophiles:
reproductive interference and
parasite specialization in *Artemia***

Soutenue le 12 décembre 2016 devant le jury composé de

Mme Ellen DECAESTECKER, Prof., Katholieke Universiteit Leuven	Rapporteur
Mme Meghan DUFFY, Prof., University of Michigan	Rapporteur
Mme Alison DUNCAN, CR, Université de Montpellier	Examineur
M. Thierry RIGAUD, DR, Université de Bourgogne	Président & Examineur
M. Thomas LENORMAND, DR, CEFE-CNRS	Directeur de thèse
M. Yannis MICHALAKIS, DR, MIVEGEC-CNRS	Codirecteur de thèse



BIOTIC CHALLENGES FOR EXTREMOPHILES:
REPRODUCTIVE INTERFERENCE
AND PARASITE SPECIALIZATION IN *ARTEMIA*

EVA J. P. LIEVENS

Doctoral dissertation prepared in the research group CEFÉ-CNRS,
under the supervision of Thomas Lenormand and Yannis Michalakis,
and submitted to the University of Montpellier in December 2016.

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Aan Kilian J. A., Bruno G. M, Anik C. B., en Dirk A. J.

TABLE OF CONTENTS

Introduction.....	5
Section 1: Reproductive interference.....	15
<i>Overview</i>	<i>15</i>
<i>Chapter 1: Maladaptive sex ratio adjustment in the invasive brine shrimp Artemia franciscana</i>	<i>17</i>
<i>Chapter 2: Discrimination against heterospecific and non-receptive females during pre-copulatory mate guarding in Artemia</i>	<i>33</i>
<i>Discussion & perspectives</i>	<i>47</i>
Section 2: Parasite specialization.....	49
<i>Overview</i>	<i>51</i>
<i>Chapter 3: Cryptic and overt host specificity shapes the epidemiology of two sympatric microsporidian species</i>	<i>53</i>
<i>Chapter 4: Infectivity, virulence and transmission in a two-host, two-parasite system..</i>	<i>71</i>
<i>Chapter 5: Specialized host exploitation constrains the evolution of generalism in microsporidian parasites of Artemia</i>	<i>115</i>
<i>Discussion & perspectives</i>	<i>143</i>
Conclusion	149
References.....	149
Appendices.....	171
<i>Appendix 1: Survival of microsporidian spores at cold temperatures & spore dormancy</i>	<i>171</i>
<i>Appendix 2: Colonization of Artemia franciscana by the cestode Flamingolepis liguloides.....</i>	<i>175</i>
<i>Appendix 3: Relationships between fitness, infectivity, virulence, and spore production in the serial passage experiment</i>	<i>179</i>
<i>Appendix 4: Résumé de la thèse en français</i>	<i>185</i>

INTRODUCTION

INTRODUCTION

1. EVOLUTION OF INTERSPECIFIC INTERACTIONS

In nature, populations are faced with a suite of abiotic and biotic challenges. Much of the research in evolutionary ecology has focused on abiotic factors (e.g. Kettlewell 1961, Macnair 1997, Huey et al. 2000, Reznick and Ghalambor 2001a, Jørgensen et al. 2007, Sexton et al. 2009a, Powles and Yu 2010, Turcotte et al. 2012, Milesi et al. 2016). However, a large part of an organisms' environment is determined by its interactions with other species: competitors, predators or prey, hosts or parasites.

Biotic challenges can have important impacts on the evolutionary trajectory of populations. Competing species can evolve to partition their niches (Brown and Wilson 1956, Lawrence et al. 2012a). Prey species evolve to evade predation (e.g. Benson 1972, Reznick and Endler 1982, Hairston and Dillon 1990, Freeman and Byers 2006) while predators adapt to these strategies (e.g. Hairston Jr. et al. 1999, Grant and Grant 2002). All species must invest in defense against parasites (e.g. Duffy and Sivars-Becker 2007, Zbinden et al. 2008), with parasites in turn adapting to exploit their hosts (Ebert 1998), potentially leading to antagonistic coevolution (Buckling and Rainey 2002, Decaestecker et al. 2007, Hall et al. 2011). In addition, evolutionary responses to biotic challenges can have cascading effects in the entire community (e.g. Lawrence et al. 2012b, Pantel et al. 2015).

The importance of interspecific interactions can easily be appreciated when species are exposed to a new biotic context. Invasions, for example, often trigger bouts of rapid evolution (reviewed by e.g. Cox 2004, Strauss et al. 2006, Shine 2012). Interspecific interactions can cause a large part of this (mal)adaptation: multiple studies have demonstrated the adaptation of invasive or invaded species to, amongst others, new hosts (e.g. Filchak et al. 2000, Carroll et al. 2005), new parasites (e.g. Hufbauer 2001, Jarvi et al. 2001, Wendling and Wegner 2015), or new prey (e.g. Shine 2012). Situations like these often prove serendipitous for the understanding of biotic factors, as they allow us to study the establishment of interspecific interactions and track their subsequent evolution (Mooney and Cleland 2001, Sax et al. 2007).

In general, however, the impact of biotic selection pressures on natural populations is not well understood (Strauss et al. 2006, Sexton et al. 2009b, Gilman et al. 2010, Alberto et al. 2013). This is largely due to the complexity of natural systems, which tend to have a prohibitive number of interspecific interactions; the problem is exacerbated by the possibilities of indirect effects and diffuse coevolution (interactions affected by the presence of other species, Strauss et al. 2005, Barraclough 2015). As a result, getting a realistic sense of the numerous selection pressures faced by populations in a natural setting is incredibly difficult. One possible solution to this problem is to study ecosystems that host a limited number of species (Strauss 2014).

Such systems allow for a comprehensive understanding of the biotic context, and offer a unique opportunity to investigate the many facets of adaptation in natural populations.

2. STUDYING INTERSPECIFIC INTERACTIONS IN THE SALTERN OF AIGUES-MORTES

Over the course of my PhD, I studied interspecific interactions in a simple, extreme environment: the commercial saltern of Aigues-Mortes in Southern France. The saltern consists of 10 000 hectares of interconnected basins, between which water is allowed to flow as a function of the salt production process. T. Lenormand and team have been studying the dominant species in this environment, the brine shrimp (*Artemia*), since 2002.

The Aigues-Mortes system has two characteristics that make it ideally suited to the study of biotic selection pressures. First, it is simple in its extremity. Saltern communities are dominated by one inescapable abiotic factor: salt. Salinities in the Aigues-Mortes saltern range from ± 50 to 250 ppt (roughly 1.5 to 7 times as salty as the average ocean), and are usually well above 100 ppt (Lievens et al. in prep.a). These extremely high salt concentrations only permit specifically adapted halophile species to establish themselves, leading to simple, tractable species communities. Second, the Aigues-Mortes saltern, which originally hosted only one *Artemia* species, has been invaded by a second *Artemia* species (discussed in detail below). With the invasive *Artemia* came a suite of novel interspecific interactions. The invasion's timing and source is known, providing us with an excellent opportunity to study the establishment and development of the new interspecific interactions.

Dramatis personae

In this section, I discuss the key inhabitants of the Aigues-Mortes ecosystem: the brine shrimp *Artemia*, their most prevalent (recorded) parasites, and the various algae, archaea and bacteria that they feed upon and live with.

Besides these groups, the saltern contains very high concentrations of viruses, about which very little is known (Santos et al. 2012), and a handful of – mostly avian – predators that forage on the *Artemia*. The most prominent of the predators is the Greater Flamingo, *Phoenicopterus roseus*, which can consume thousands of *Artemia* per day (Deville 2013); even this high rate of consumption does not make an appreciable dent in the *Artemia* population (personal observation), which is more likely to be regulated by environmental factors (food availability, Browne 1980, salinity, Wear and Haslett 1986, temperature, Barata et al. 1996a).

The brine shrimp Artemia parthenogenetica and Artemia franciscana

Artemia (Branchiopoda: Anostraca), also called brine shrimp, is a genus of small branchiopod crustaceans belonging to the order Anostraca. *Artemia* are renowned for their extreme salinity tolerance, able to survive in brine of up to 250-300 ppt (Van Stappen 2002, Nougue et al. 2015).

Different strains can also tolerate a wide range of ionic compositions, including chloride, sulfate, or carbonate waters (Cole and Brown 1967, Van Stappen 2002). With these abilities, brine shrimp have populated salt lakes and commercial salt pans around the globe (Fig. 1).

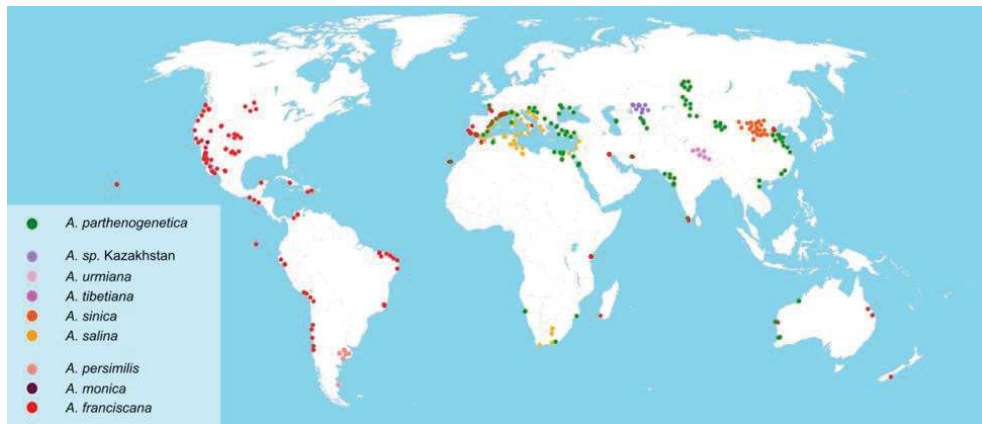


Figure 1. Global distribution of the genus *Artemia*. *A. parthenogenetica* is the only asexual clade, all others are sexual. Any *A. franciscana* occurring in the Old World are introduced or invasive. Figure modified with permission from Noug   (2015), who collated information collected by Van Stappen (2002) and Mu  oz & Pacios (2010).

In the saltern of Aigues-Mortes, two species of brine shrimp occur in sympatry: *Artemia parthenogenetica*, and *Artemia franciscana*. *A. parthenogenetica* is a native, parthenogenetic clade that is spread throughout the Old World (green dots in Fig. 1) (Mu  oz et al. 2010). *A. franciscana* is a sexual species native to the New World (red dots in Fig. 1) (Thi  ry and Robert 1992, Amat et al. 2005). In Aigues-Mortes, as elsewhere, *A. franciscana* was introduced for commercial purposes (Amat et al. 2005, Rode et al. 2013c). The Aigues-Mortes population originates in repeated introductions of cysts from the San Francisco Bay, California, USA (1970-1979), with an additional introduction of cysts collected in the Great Salt Lake, Utah, USA (1979, Rode et al. 2013c); it is likely that admixture between these populations has occurred since (Mu  oz et al. 2014). In its native range, *A. franciscana* has no sympatric congeners (Fig. 1). *A. franciscana* and *A. parthenogenetica* are genetically distinct and cannot interbreed (Macdonald and Browne 1987).

Artemia have a short-lived, iteroparous life history. Individuals mature within a few weeks, reaching adult sizes of 0.5 to slightly over 1 cm (Rode et al. 2013c), and have a life expectancy of about 3 months (Browne and Sallee 1984). Adult females of most *Artemia* species produce a clutch of tens of offspring every \pm 5 days (Browne and Sallee 1984). Under ideal conditions, these clutches contain live larvae, called ‘nauplii’; if the female is stressed, the clutches contain diapausing eggs, called ‘cysts’, which can be stored for decades (Lenz and Browne 1991).

The *Artemia* population in Aigues-Mortes is highly seasonal. *A. parthenogenetica* is present from spring to fall, with highest densities in late summer, and overwinters in cyst form (Macdonald and Browne 1989, Rullman et al. personal communication Lievens et al. 2016). *A. franciscana* is

present year-round, but its densities are also low in winter (Thiéry and Robert 1992, Rullman et al. personal communication Lievens et al. 2016). Altogether, the yearly *Artemia* population is estimated to be on the order of 10^9 - 10^{10} (extrapolated from the exploitation data of the commercial *Artemia* fishery Camargue Pêche, Grau-du-Roi, France, F. Gout personal communication).

Despite the regular isolation of parts of the Aigues-Mortes saltern for the purpose of salt production, the flow of water between basins is frequent enough to maintain genetic homogeneity within the *Artemia* populations (Nougué et al. 2015).

Microsporidian and helminth parasites

Among the many parasites infecting *A. franciscana* and *A. parthenogenetica* (e.g. yeasts, Butinar et al. 2005, microsporidians, Ovcharenko and Wita 2005, helminths, Sánchez et al. 2013b, Georgiev et al. 2014), three species have dominated our recent research efforts: the cestode *Flamingolepis liguloides* and the microsporidians *Anostracospira rigaudi* and *Enterocytozpora artemiae* (Sánchez et al. 2012, Rode et al. 2013c, 2013b, 2013a). *F. liguloides* (Cestoda: Cyclophyllidae) is a trophically transmitted parasite of the Greater Flamingo, the most important predator of *Artemia* in Aigues-Mortes (see below). It uses *Artemia* as an intermediate host (Gabrion et al. 1982), and its phenotypic effects on this host include changes in color (Amat et al. 1991a, Sánchez et al. 2006), castration and longer lifespan (Amat et al. 1991a), surfacing behavior (Thiéry et al. 1990, Sánchez et al. 2007, though see Rode et al. 2013b), and the induction of swarming (Rode et al. 2013b). *A. rigaudi* and *E. artemiae* (Microsporidia) are horizontally transmitted parasites of *A. franciscana* and *A. parthenogenetica*. Both microsporidians infect the gut epithelium, transmitting infection through spores released with the feces (Rode et al. 2013a). These species were only recently described, and much less is known about their biology: they induce depth-dependent swarming behavior (Rode et al. 2013b), and *A. rigaudi* is suspected to reduce the clutch rate of female hosts (Rode et al. 2013c).

A. parthenogenetica is the historical host of both *A. rigaudi* and *F. liguloides*. Both parasites are native to the Old World (*A. rigaudi* has been detected in France and Ukraine, Rode et al. 2013c; *F. liguloides* occurs throughout the Mediterranean basin, see Appendix 2), and neither has ever been detected in *A. franciscana*'s native range (Rode et al. 2013c, Redón et al. 2015b).

Accordingly, the prevalences recorded for each are much higher in *A. parthenogenetica* than in *A. franciscana* (*A. rigaudi*: ± 60 vs. $\pm 15\%$ Rode et al. 2013c; *F. liguloides*: ≤ 83 vs. $\leq 2\%$, see Appendix 2).

For *E. artemiae*, it is unclear which *Artemia* population represents its historical host. *E. artemiae* has been detected both in the New World (in *A. franciscana* populations, Rode et al. 2013c, Lievens et al. unpublished data) and in *A. franciscana*-invaded areas of the Old World (though sampling was fragmentary, Rode et al. 2013c). Rode et al. detected one single nucleotide polymorphism (SNP) difference between Old and New World *E. artemiae*, but they

recognized that this apparent difference could be due to inadequate sampling, and were unable to conclude whether *E. artemiae* co-invaded with *A. franciscana*, invaded from the New World at an earlier time, or was already present as a cosmopolitan species. It is also possible that *E. artemiae* invaded from a separate Old World *Artemia* population. However, some further indication that *A. franciscana* is *E. artemiae*'s historical host is given by the microsporidian's higher prevalence in this host compared to *A. parthenogenetica* ($\pm 45\%$ vs. $\pm 15\%$, Rode et al. 2013c).

All three parasites infect their hosts after ingestion (Robert and Gabrion 1991, Rode et al. 2013a); because *Artemia* are non-selective filter feeders (Reeve 1963a), the pool of transmissible stages is shared between *A. franciscana* and *A. parthenogenetica* (cf. Fels 2006). Unsurprisingly, given this mode of transmission, the three parasites regularly coinfect. The available field data has not revealed any facilitation or inhibition effects on the establishment of coinfections, nor any robust effects of coinfection on the host phenotype (Rode et al. 2013b). Overall, *A. franciscana* seems to be less affected by the local parasites than is *A. parthenogenetica*, suggesting that its success as an invader is at least partly due to enemy release (Sánchez et al. 2012, Rode et al. 2013c, Redón et al. 2015a).

Unicellular algae and the bacteria to digest them

The diet of *Artemia* is mostly made up of unicellular algae (Lenz and Browne 1991). In order to successfully digest these algae, *Artemia* are dependent on the establishment of a gut microbiome, consisting of halophile bacteria from the environment that establish themselves in the *Artemia* gut after the (axenic) cysts hatch (Nougue et al. 2015). Not much is known about these microbiota, nor about their interactions with *Artemia*'s various parasites.

Interspecific interactions

The key interactions between the *Artemia*, parasites, and microbiota that drive the Aigues-Mortes ecosystem are pictured in Fig. 2 (pre-invasion interactions by dotted lines, post-invasion interactions by solid lines), and summarized below.

- 1. Competition between the two *Artemia* species.** *A. franciscana* and *A. parthenogenetica* compete for resources (demonstrated by e.g. Browne 1980, Browne and Halanych 1989, Lenz and Browne 1991), which may have led to adaptive niche shifts for either species after the invasion of *A. franciscana* (Brown and Wilson 1956). At the moment, the two species are somewhat segregated, with *A. parthenogenetica* dominating in summer and *A. franciscana* in fall through spring (Lievens et al. 2016); this may or may not be due to previous niche partitioning (the “ghost of competition past”, Connell 1980).
- 2. Reproductive interference.** If *A. franciscana* are ancestrally unable to distinguish con- from heterospecific females, reproductive interference by *A. parthenogenetica* should

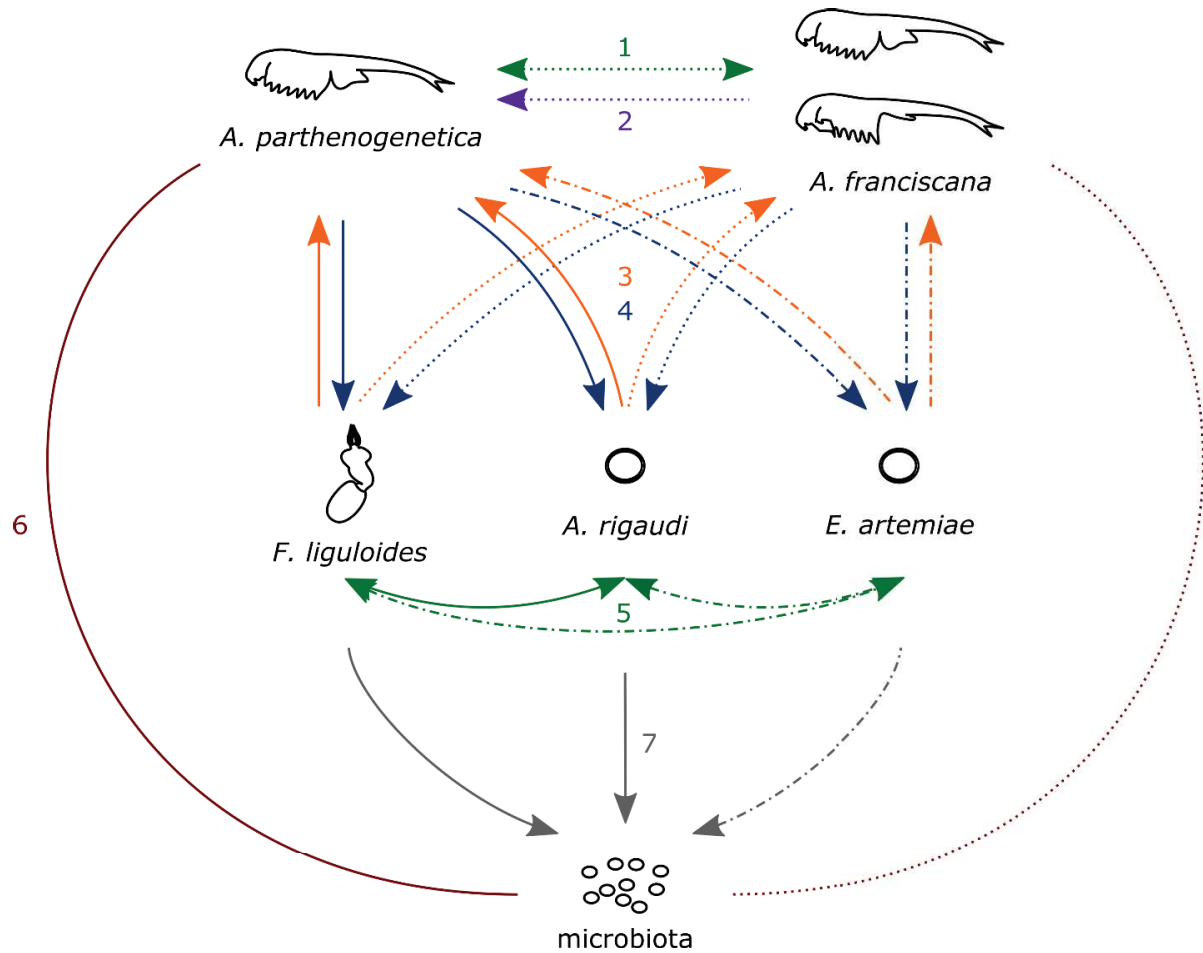


Figure 2. Key interspecific interactions in the post-invasion *Artemia* system of Aigues-Mortes. Solid lines represent interactions that were present before the invasion of *A. franciscana*; dotted lines represent novel post-invasion interactions; dot-dashed lines represent interactions for which we cannot determine whether they are ancestral or novel. Arrow colors represent the type of interaction: green (1 & 5), competition; purple (2), reproductive interference; orange (3), parasitism; blue (4), host resistance; dark red (6), commensalism; gray (7), potential host-mediated effects. Arrows point in the direction of potential ‘adaptation to’, e.g. arrow 2 has *A. franciscana* adapting to the presence of *A. parthenogenetica*. Numbers refer to the list of interactions in the text. Female and male *Artemia* are represented for *A. franciscana*, respectively top and bottom.

lead to the evolution of species discrimination (Gröning and Hochkirch 2008). *A. parthenogenetica* females, in turn, may suffer from harassment by *A. franciscana* males.

3. **Parasitism.** The availability of new host resources for the native and invasive parasites has led to extensions of the host range for the microsporidian parasites (Rode et al. 2013c), who might now be expected to optimize their exploitation of the two hosts (Regoes et al. 2000, Gandon 2004). *F. liguloides*, in contrast, has never been reported to infect *A. franciscana* in Aigues-Mortes (Sánchez et al. 2012, Rode et al. 2013b), and may therefore still be expected to colonize the new host.
4. **Host resistance.** Exposure of the *Artemia* hosts to the novel parasites should impose selection for host defenses (Duffy and Sivars-Becker 2007, Zbinden et al. 2008).
5. **Competition between parasites.** Competition between parasite species for host resources is common, and can lead to evolutionary changes in parasite life history or niche breadth (Alizon et al. 2013). The arrival of *A. franciscana* and *E. artemiae* may have changed the competitive dynamics, especially given the similarity of the two microsporidians.
6. **Commensalism.** *Artemia* are dependent on local microbiota for digestion (Nougue et al. 2015).
7. **Potential host-mediated facilitation or inhibition effects.** In several host-parasite systems, microbiota have been shown to affect the immune system of the host, or the establishment and virulence of parasites (Dillon and Dillon 2004, Oliver et al. 2010, Koch and Schmid-Hempel 2012). This may be occurring in *Artemia* as well.

It should be kept in mind that these interactions do not exist in a vacuum, but in a complex ecological network. They almost certainly have indirect effects on each other and on the demography of the two *Artemia* species, which will feed back into the interactions themselves. For example, adaptation of *F. liguloides* to *A. franciscana* could lead to higher population sizes of *F. liguloides*, which could increase the cestode burden on *A. parthenogenetica* (parasite spillback, Kelly et al. 2009), which could select for stronger resistance to this parasite, or higher virulence in one of the competing microsporidians (Alizon et al. 2013, Fenton 2013). However, the ecosystem is simple enough that we can confidently identify the major interactions - we may have missed the effects of some viruses, for example, but we can certainly exclude the possibility of other competitors for *Artemia* or other hosts for their parasites (confirmed by Rode et al. 2013a).

Interspecific interactions addressed in this dissertation

Over the course of my PhD, I studied three of the seven interspecific interactions described in Fig. 2, taking liberal advantage of the opportunities offered by the invasion of *A. franciscana*. I

list them briefly here; more context and background information can be found in the relevant sections and chapters.

2. **Reproductive interference.** The evolution of invasive *A. franciscana*'s reproductive behavior in the presence of *A. parthenogenetica* is addressed in Section 1 (Chapters 1 & 2).
3. **Exploitation of host resources.** The specificity of *A. rigaudi* and *E. artemiae* in the expanded two-host system, and their response to selection for generalism and specialism, is addressed in Section 2 (Chapters 3-5). The colonization of the novel host *A. franciscana* by *F. liguloides* is briefly discussed in Appendix 2.
4. **Host resistance.** The resistance of *A. franciscana* and *A. parthenogenetica* to their novel parasites is addressed in Section 2 (Chapter 4). The maintenance of *A. franciscana*'s resistance to *F. liguloides* is briefly discussed in Appendix 2.

SECTION 1:

REPRODUCTIVE INTERFERENCE

OVERVIEW

Context

For many related, sympatric species, species recognition during reproductive behavior is a key adaptation to the risk of pursuing, mating, or hybridizing with a heterospecific (Coyne and Orr 1989, Futuyma 2009 chap. 17, Ivens et al. 2009). When previously allopatric species encounter each other, species recognition may go awry. The lack of recognition can lead to reproductive interference, “any kind of interspecific interaction during the process of mate acquisition that adversely affects the fitness of at least one of the species involved and that is caused by incomplete species recognition” (Gröning and Hochkirch 2008). Because it is costly, reproductive interference is expected to select for improved species recognition or other premating barriers; this has been demonstrated in e.g. recently-sympatric species of *Drosophila* (Coyne and Orr 1989) and damselflies (Mullen and Andrés 2007).

In this section

In Aigues-Mortes, the invasive species *A. franciscana* can be expected to suffer from reproductive interference. *A. franciscana* does not have sympatric congeners in its native range, so it is unlikely to be pre-adapted to distinguish between conspecific females and asexual *A. parthenogenetica* females. Although the two *Artemia* species do not hybridize (Macdonald and Browne 1987), *A. franciscana* males have time-intensive mate guarding behaviors (Belk 1991), and – as will be seen – *A. franciscana* females make sex allocation decisions based on the adult sex ratio. If *A. parthenogenetica* females trigger the same responses as conspecific females, these behaviors should become costly, and select for the rapid evolution of species discrimination.

This section investigates the reproductive interference of *A. franciscana* by *A. parthenogenetica* females, covering two behaviors: sex allocation and mate guarding.

- **Chapter 1: Maladaptive sex ratio adjustment in the invasive brine shrimp *Artemia franciscana*.** Prompted by field observations, we show that *A. franciscana* females adjust their offspring sex ratio as a function of the adult sex ratio in the population, mistakenly including *A. parthenogenetica* females in their estimate of the latter. By comparing this behavior with that of native-range *A. franciscana*, we further demonstrate that there has not (yet) been any evolution towards species discrimination.
- **Chapter 2: Discrimination against heterospecific and non-receptive females during pre-copulatory mate guarding in *Artemia*.** In this chapter, we demonstrate that invasive *A. franciscana* males can discriminate between con- and heterospecific females when mate guarding. Future work will determine if this is the result of pre-adaptation or of evolved species discrimination in the invasive population.

CHAPTER 1

MALADAPTIVE SEX RATIO ADJUSTMENT IN THE INVASIVE BRINE SHRIMP *ARTEMIA FRANCISCANA*

Eva J. P. Lievens, Gil J. B. Henriques, Yannis Michalakis, Thomas Lenormand

Sex allocation theory is often hailed as the most successful area of evolutionary theory due to its striking success as a predictor of empirical observations. Most naturally occurring sex ratios can be explained by the principle of equal investment in the sexes or by cases of ‘extraordinary’ sex allocation. Deviations from the expected sex ratio are often correlated with weak selection or low environmental predictability; true cases of aberrant sex allocation are surprisingly rare. Here, we present a case of long-lasting maladaptive sex allocation, which we discovered in invasive populations of the exclusively sexual brine shrimp *Artemia franciscana*. *A. franciscana* was introduced to Southern France roughly 500 generations ago; since then it has coexisted with the native asexual species *Artemia parthenogenetica*. Although we expect *A. franciscana* to produce balanced offspring sex ratios, we regularly observed extremely male-biased sex ratios in invasive *A. franciscana*, which were significantly correlated to the proportion of asexuals in the overall population. We experimentally proved that both invasive- and native-range *A. franciscana* overproduced sons when exposed to excess females, without distinguishing between conspecific and asexual females. We conclude that *A. franciscana* adjust their offspring sex ratio based on the adult sex ratio, but are information limited in the presence of asexual females. Their facultative adjustment trait, which is presumably adaptive in their native range, has thus become maladaptive in the invasive range where asexuals occur. Despite this, it has persisted unchanged for hundreds of generations.

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Maladaptive Sex Ratio Adjustment in the Invasive Brine Shrimp *Artemia franciscana*

Eva J.P. Lievens,^{1,2} Gil J.B. Henriques,¹ Yannis Michalakis,² and Thomas Lenormand^{1,*}

¹UMR 5175 CEFÉ, CNRS–Université de Montpellier–Université P. Valéry–EPHE, 1919 Route de Mende, 34293 Montpellier Cedex 5, France

²UMR 5290 MIVEGEC, CNRS–IRD–Université de Montpellier, 911 Avenue Agropolis BP 64501, 34394 Montpellier Cedex 5, France

*Correspondence: thomas.lenormand@cefe.cnrs.fr

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SUMMARY

Sex allocation theory is often hailed as the most successful area of evolutionary theory due to its striking success as a predictor of empirical observations [1]. Most naturally occurring sex ratios can be explained by the principle of equal investment in the sexes [2–4] or by cases of “extraordinary” sex allocation [5]. Deviations from the expected sex ratio are often correlated with weak selection or low environmental predictability (e.g., [6, 7]); true cases of aberrant sex allocation are surprisingly rare [8]. Here, we present a case of long-lasting maladaptive sex allocation, which we discovered in invasive populations of the exclusively sexual brine shrimp *Artemia franciscana*. *A. franciscana* was introduced to Southern France roughly 500 generations ago [9]; since then, it has coexisted with the native asexual species *Artemia parthenogenetica* [10]. Although we expect *A. franciscana* to produce balanced offspring sex ratios, we regularly observed extremely male-biased sex ratios in invasive *A. franciscana*, which were significantly correlated to the proportion of asexuals in the overall population. We experimentally proved that both invasive- and native-range *A. franciscana* overproduced sons when exposed to excess females, without distinguishing between conspecific and asexual females. We conclude that *A. franciscana* adjust their offspring sex ratio in function of the adult sex ratio but are information limited in the presence of asexual females. Their facultative adjustment trait, which is presumably adaptive in their native range, has thus become maladaptive in the invasive range where asexuals occur. Despite this, it has persisted unchanged for hundreds of generations.

RESULTS

Although we expect sexual *Artemia* to produce balanced offspring sex ratios (see the [Experimental Procedures](#)), we regularly observed extremely male-biased sex ratios in *Artemia franciscana* in Southern France, and these biases were strongly correlated with the proportion of *Artemia parthenogenetica* in

the sample (Figure 1). We collected demographic data from 56 samples of *Artemia* (over 7,000 adults sexed) taken from 12 sampling sites in two large salterns between the years 2010 and 2015 and analyzed the factors affecting the sex ratio of *A. franciscana* in each sample (sex ratio r = number of adult males / total number of adults). The proportion of *A. parthenogenetica* in the samples varied seasonally between 0 and 0.99 (Figure S2). The sex ratio of *A. franciscana* varied very strongly among samples and throughout the year ($0.4 \leq r \leq 1$), with the highest sex ratios occurring in December and the lowest in May (Figure 1). After controlling for the seasonal effect, the sex ratio of *A. franciscana* was positively and strongly correlated with the proportion of *A. parthenogenetica* (Figure 1; Table S1).

Our field results suggested that the presence of *A. parthenogenetica* causes the adult sex ratio of *A. franciscana* to become male biased, and we designed an experiment to confirm this. We combined different numbers of lab-raised *A. franciscana* and *A. parthenogenetica* into parental groups (Table 1; for a full explanation of the tested hypotheses, see the [Experimental Procedures](#)) and monitored their offspring sex ratio (3,820 offspring sexed; Table S2). First, we verified that *A. franciscana* from Southern France (the invasive range) produced balanced offspring sex ratios when placed in groups with balanced adult sex ratios. This was indeed the case, independently of the total size of the group (Figure 2, “invasive range” and “invasive range*” groups). We then tested whether the biased sex ratios observed in the field could be induced by adding asexual females to the group and, if so, whether addition of conspecific females elicited the same response. We found that when the adult sex ratio was female biased, whether by the addition of *A. parthenogenetica* or *A. franciscana* females, *A. franciscana* females produced male-biased offspring sex ratios (Figure 2, “invasive range + ♀” and “invasive range + P” groups). Finally, we investigated whether sex ratio adjustment in the presence of asexual females was a trait that *A. franciscana* had acquired in its invasive range, or whether it was an ancestral trait that could be observed in native populations. The latter was true: when we placed *A. franciscana* from a native-range population (Great Salt Lake, Utah) in groups with “excess” *A. parthenogenetica* females, they also produced biased offspring sex ratios (Figure 2, “native range + P” group). Statistically, the three female-biased parental groups produced equivalent male-biased offspring sex ratios (0.60, 0.62, and 0.58, respectively; Table S2), which differed from the balanced offspring sex ratios produced by the equal sex ratio groups (0.51 and 0.48; Table S2; statistical comparison in Table S3).



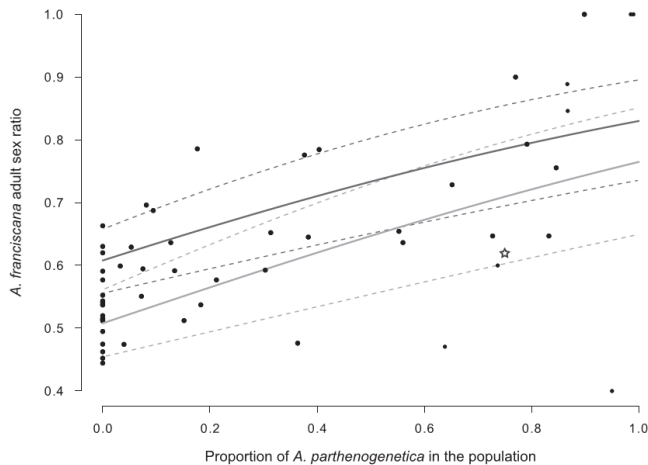


Figure 1. The Adult Sex Ratio of *A. franciscana* in Natural Populations Increases When Asexual *A. parthenogenetica* Are Present

The sex ratio also varies with month; the curves represent the predictions of the best model for December (highest sex ratios; shown in dark gray) and May (lowest sex ratios; shown in light gray), and the dashed lines represent the upper and lower binomial 95% CIs for the two curves. Small points represent the samples where fewer than 20 *A. franciscana* were counted. The star corresponds to our experimental result for the treatment “invasive range + P” (Figure 2). See also Figure S2.

DISCUSSION

The first conclusion that we draw from our experimental results is that *A. franciscana* can plastically adjust their offspring sex ratio in response to changes in the adult sex ratio (Figure 2, compare the first three treatments). This could be advantageous in their native range: Werren and Charnov [11] proposed that in species with overlapping generations, perturbations of the stable age distribution could select for facultative sex ratio adjustment; parents should then produce more offspring of the under-represented, more competitive sex. Although it is unclear to which extent *A. franciscana* generations overlap in nature [12], the native populations do regularly experience perturbations in the adult sex ratio (see Figure S1 and Table S4), and our results confirm their ability to respond to these perturbations. It is worth noting that females are the heterogametic sex in *A. franciscana* [13, 14] and that the sex-determining meiosis in oocytes is triggered by fertilization [15]; this provides females with the potential to adjust the primary sex ratio in real time [16–18]. This study pro-

vides the first evidence of facultative sex ratio adjustment in *Artemia*, which may become a useful model organism to resolve some of the empirical debate around Werren and Charnov’s model (e.g., [19, 20]).

Second, our experiment confirms the field data and shows that *A. franciscana* mistakenly adjust their offspring sex ratio in response to the presence of *A. parthenogenetica* females (Figure 2, compare the third and fourth treatments). It is likely that *A. franciscana* are unable to differentiate between con- and heterospecific females; this has already been noted with regards to male mate choice [21, 22] and is unsurprising because it is the only brine shrimp species in its native range [10]. In the invasive range, therefore, *A. franciscana* seem to be limited by information: they mistake parthenogenetic females for conspecifics. A handful of previous studies have described similar effects of information constraints on sex ratio adjustments [23–25], but these studies deal with spatially segregated patches (anthills, fly pupae) where information is not universally faulty and the population-wide impact of bad decisions may be limited. In contrast, where *A. franciscana* coexists with *A. parthenogenetica*, mistaken sex ratio adjustment occurs on a population scale. Interestingly, both *A. franciscana* and *Nasonia* [25] females make poor sex allocation decisions based on a failure to identify heterospecifics; such constraints, if looked for, may be more common than we think in sex-ratio-adjusting species.

Facultative sex ratio adjustment is thus maladaptive for *A. franciscana* in its invasive range. When *A. parthenogenetica* are present, the false sex allocation cue they cause overwhelms any relevant variation in *A. franciscana* sex ratio and causes a mass overproduction of males. Females will then become the more reproductively competitive sex, and parents mistakenly producing excess sons will be disadvantaged compared to any (hypothetical) parent producing fewer sons in favor of daughters [2, 11]. This disadvantage is unlikely to be compensated for by “correct” sex ratio adjustment at other points in the lifespan, as *A. parthenogenetica* are typically present from May until October, well above the life expectancy of *Artemia* [26].

Finally, and surprisingly, invasive *A. franciscana* in Southern France still display the exact same sex ratio adjustment response as naive populations, despite ~500 generations of exposure to an environment where this behavior is costly (Figure 2, compare the fourth and fifth treatments). This result contrasts sharply with previous experimental evolution studies on sex allocation, which show that populations can evolve to match predicted sex ratios within 2 to 54 generations [27–31] (though at least one natural

Table 1. Composition of the Experimental Treatments

Treatment	Parental Group Composition				Origin of <i>A. f.</i>	Parental Sex Ratio	
	<i>A. f.</i> Males	<i>A. f.</i> Females	<i>A. p.</i> Females	Total		“True”	“Perceived”
Invasive range	5	5	—	10	A-M, France	0.5	0.5
Invasive range*	20	20	—	40	A-M, France	0.5	0.5
Invasive range + ♀	5	35	—	40	A-M, France	0.125	0.125
Invasive range + P	5	5	30	40	A-M, France	0.5	0.125
Native range + P	5	5	30	40	GSL, USA	0.5	0.125

We formed parental groups out of different numbers of *A. franciscana* (*A. f.*) and *A. parthenogenetica* (*A. p.*), with different “true” and “perceived” parental sex ratios. “True” sex ratio, *A. franciscana* males / *A. franciscana* males and females; “perceived” sex ratio, *A. franciscana* males / (*A. franciscana* males and females + *A. parthenogenetica*). A-M, Aigues Mortes; GSL, Great Salt Lake.

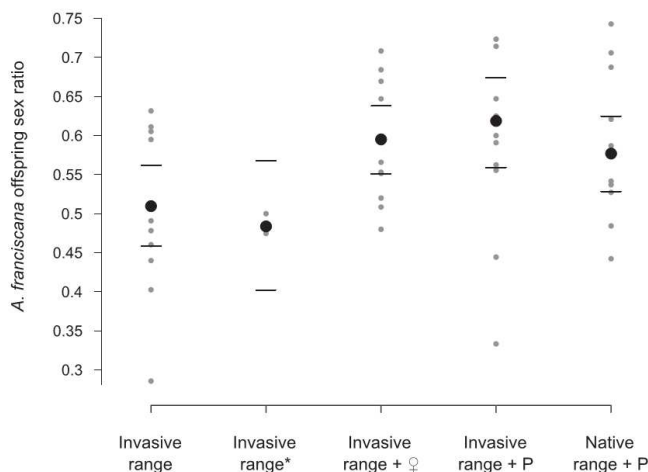


Figure 2. *A. franciscana* Produce Male-Biased Offspring Sex Ratios When Experimentally Exposed to Female-Biased Parental Sex Ratios

They do this independently of their geographical origin (“invasive range” versus “native range”) and of whether the female bias is caused by conspecific females (“+♀”) or by *A. parthenogenetica* asexuals (“+P”). Small gray points are the results for each replicate, large black points are the mean, and horizontal bars represent the binomial 95% confidence intervals. See also Table S2.

population has not evolved within this timeframe [32]). Why is adaptation so much slower in invasive *A. franciscana*? Selection should be strong, so that even a loss-of-function mutation restoring fixed Mendelian segregation of the Z and W chromosomes to oocytes—and thus a Fisherian sex ratio—should generate a considerable net advantage. For example, based on our experimental treatment “invasive range + P,” we estimate that in a stable population containing 75% asexuals, the relative fitness of a mutant Fisherian parent would be $W = 1.06$ compared to a typical parent producing 62% males [3]. The occurrence of such a loss-of-function mutation is unlikely to be limiting because the Southern French *Artemia* population is very large (estimated on the order of 10^9 – 10^{10} in the Aigues-Mortes saltern alone by commercial exploiter F. Gout, Camargue Pêche, Grau-du-Roi, France; unpublished data), so that we expect Nu to be large. The persistence of maladaptation may be explained by the requirement of a more complex series of mutations; alternatively, and more plausibly, the necessary loss-of-function mutation may be present but (partially) recessive. Supporting the latter possibility, a recessive loss of plastic sex allocation has recently been shown in parasitoid *Asobara japonica* wasps [33]. If the mutation is recessive, the large population size of *A. franciscana* could slow its increase to a detectable frequency [34, 35].

The evolution of an optimal sex allocation in invasive *A. franciscana* has thus been limited by two factors. First, a previously beneficial sex ratio adjustment trait became information limited when the species found itself in a novel ecological situation (sympatry with *A. parthenogenetica*). Afterward, as discussed above, it is likely that genetic limitations slowed the loss of the now-maladaptive trait.

Hamilton famously said that the study of sex allocation “best proves [...] the Neodarwinism paradigm as a whole” [1], due to

its success in demonstrating adaptation. However, adaptation is only one side of the evolutionary coin: evolution proceeds locally by tinkering with the available variability and without foresight, so that maladaptation is also expected [36, 37]. Indeed, it is perhaps surprising that so few cases of maladaptation have been reported in this well-studied field. The extraordinary maladaptive sex ratios of invasive *A. franciscana*, perhaps along with other “inexplicable” sex ratios (e.g., [8]), show that like any other trait shaped by natural selection, sex allocation cannot always be optimal.

EXPERIMENTAL PROCEDURES

Biology of *Artemia* and the Invasion of *A. franciscana*

The brine shrimp *Artemia* (Branchiopoda: Anostraca) is a genus of small crustaceans present worldwide in hypersaline environments, notably salt lakes and salt pans. In general, *Artemia* mature within a few weeks and have a life expectancy of ± 3 months [26]. When under optimal conditions, females of most *Artemia* species produce a large clutch of ovoviparous offspring roughly every 5 days [26]. When stressed, *Artemia* produce diapausing eggs that can be stored for years [12].

Sexual *Artemia* populations can be expected to conform to the Fisherian prediction of a balanced sex ratio, since populations are enormous and lack spatial structure [12, 38]. The sex ratio of sexual *Artemia* hatched from diapausing eggs is indeed ~ 0.5 (Table S4), although the adult sex ratios in natural populations are often variable (Table S4), possibly due to differences in male and female tolerance of abiotic factors [39] or food quality [40].

A. franciscana is a bisexual species native to the New World, where it is not sympatric with any other *Artemia* species. The species has been widely introduced to the Old World for commercial purposes [10]. Although *A. franciscana* typically competitively excludes its native congeners, this is not the case in Southern France, where the invader still coexists with native parthenogenetic clades, traditionally called *A. parthenogenetica* [10, 41]. We estimate that this coexistence is roughly 500 generations old: the first introduction of *A. franciscana* occurred in 1970 [9], and as *A. franciscana* is present and reproductive during most of the year ([41] and unpublished data), as many as 12 (overlapping) generations may be produced over the course of a year. *A. franciscana* and *A. parthenogenetica* are genetically distinct and cannot interbreed [42].

Field Observations of *A. franciscana* Sex Ratios in the Native Range

To confirm the reportedly variable sex ratios of *A. franciscana* in its native range (see Table S4), we requested and obtained survey data of the adult sex ratio in the Great Salt Lake from the Great Salt Lake Ecosystem Program (Utah Division of Wildlife Resources). The Great Salt Lake population is one of the largest populations of *A. franciscana*’s native range [43]. The dataset contained adult sex ratios recorded from 453 field samples collected between 2000 and 2014. We excluded the years 2000–2002 from the dataset because contamination of the lake with parthenogenetic females has been reported for those years [43]; unfortunately, the proportion of the population that was parthenogenetic during this period is not known, so we were unable to calculate sex ratios. The data are represented visually in Figure S1 and have been included in Table S4.

Field Observations of *A. franciscana* Sex Ratios in the Invasive Range Sampling

We quantified the sex ratio of *A. franciscana* in its invasive range using 55 different samples from Southern France (Table S5). Samples were taken in Aigues-Mortes (43.53°N, 4.21°E; 53 samples from ten sites) and Gruissan (43.08°N, 3.09°E; two samples from two sites); some samples consisted of freshly sampled, live *Artemia*, and others had been conserved in 96% EtOH after sampling. The samples spanned the period 2010–2015 and included all seasons (March–May, 8; June–August, 19; September–November, 16; December–February, 12). For each sample, we counted the number of adult male *A. franciscana*, adult female *A. franciscana*, and adult female *A. parthenogenetica* in a representative subsample (average number

Table 2. Expectations of the Experiment under the Different Scenarios

Scenario	Expected Offspring Sex Ratio under Each Scenario			
	Invasive Range*	Invasive Range + ♀	Invasive Range + P	Native Range + P
Ancestral trait	0.5	male biased	male biased	male biased
Novel trait—discriminate trigger	0.5	0.5	male biased	0.5
Novel trait—indiscriminate trigger	0.5	male biased	male biased	0.5
Interference	0.5	0.5	male biased	male biased

Our experimental setup allowed us to distinguish between the four scenarios that explained our field results (male-biased sex ratios in the presence of *A. parthenogenetica* for invasive *A. franciscana*), as shown in the table. The scenarios and predictions are described in the [Experimental Procedures](#).

of individuals counted per sample = 132; minimum = 19; maximum = 428). *A. parthenogenetica* occasionally produce “rare” males, which may have been misclassified as male *A. franciscana*, but their frequencies are so low (almost exclusively <1% [44]) that this should not bias our estimation of the *A. franciscana* sex ratio. We calculated the sex ratio of *A. franciscana* as the proportion of males in that species; this is the tertiary or adult sex ratio.

Statistical Analysis

See the [Supplemental Experimental Procedures](#).

Experimental Test of Sex Ratio-Biasing in *A. franciscana*

Experimental Design

Based on the *A. franciscana* sex ratios that we observed in the invasive range, we hypothesized that the presence of *A. parthenogenetica* causes the adult sex ratio of *A. franciscana* to become male biased. We considered that one of three scenarios could be underlying this pattern. The first possibility was that native-range *A. franciscana* are facultative sex ratio adjusters, able to plastically adjust their offspring sex ratio in response to occasional perturbations in the adult sex ratio (as suggested by Werren and Charnov [11]). This trait would have been carried over to the invasive range, where the presence of *A. parthenogenetica* females could trigger the same response as an overabundance of conspecific females. We called this possibility the “ancestral trait” scenario. Second, in the “novel trait” scenario, invasive-range *A. franciscana* could have evolved to produce more sons in response to the presence of *A. parthenogenetica* for unknown reasons. Within this scenario, we considered that invasive-range *A. franciscana* could have evolved to react specifically to the presence of *A. parthenogenetica* (a “discriminate trigger” or to the presence of any females (an “indiscriminate trigger”). Finally, we considered the possibility that *A. parthenogenetica* manipulate their competitors into producing unnecessary males, which we called the “interference” scenario.

We tested our hypothesis and distinguished between the three underlying scenarios by tracking the offspring sex ratio produced by adult *A. franciscana* when placed in parental groups with varying sex ratios. The treatments were as follows (Table 1): “invasive range,” “invasive range + ♀,” and “invasive range + P” all used *A. franciscana* from the invasive range and had no bias, a “true” female bias caused by conspecific females, and a “false” female bias caused by parthenogenetic females, respectively. The treatment “native range + P” used *A. franciscana* from the native range and had a “false” female bias caused by parthenogenetic females. We expected different offspring sex ratios for these four treatments under the scenarios “ancestral trait,” “novel trait,” and “interference”; the predictions are shown in Table 2. Our baseline expectation was a balanced offspring sex ratio (see above). Where we expected male-biased offspring sex ratios, we were unable to predict the bias precisely: for the “ancestral trait” scenario, we would need to know to which extent generations overlap in the field [11], and we could not make theoretical predictions for the speculative “novel trait” and “interference” scenarios. Each treatment was replicated ten times. Although density was kept constant (see below), we accounted for the possibility that sex ratio might be affected by the absolute number of parental individuals by including two replicates of an additional control treatment “invasive range*,” with a higher number of individuals (Table 1).

Every 5 days over a period of 25 days, we harvested the live larvae (nauplii) produced by each parental group, counted them, and reared them in separate jars until maturity. When the nauplii reached sexual maturity, we determined their species and sex. We calculated the offspring sex ratio produced by

each parental group as the proportion of adult offspring in that group that were male (the tertiary offspring sex ratio). We calculated the offspring sex ratio produced by each parental group as the proportion of adult offspring in that group that were male (the tertiary offspring sex ratio). Some of the offspring sex ratio data were excluded due to errors during data collection.

Experimental Conditions

See the [Supplemental Experimental Procedures](#).

Statistical Analysis

See the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2016.03.074>.

AUTHOR CONTRIBUTIONS

Conceptualization, E.J.P.L., Y.M., and T.L.; Methodology, E.J.P.L., G.J.B.H., Y.M., and T.L.; Investigation, G.J.B.H., E.J.P.L., and T.L.; Formal Analysis, G.J.B.H.; Writing – Original Draft, E.J.P.L.; Writing – Review & Editing, E.J.P.L., Y.M., and T.L.; Supervision, Y.M. and T.L.; Funding Acquisition, T.L.

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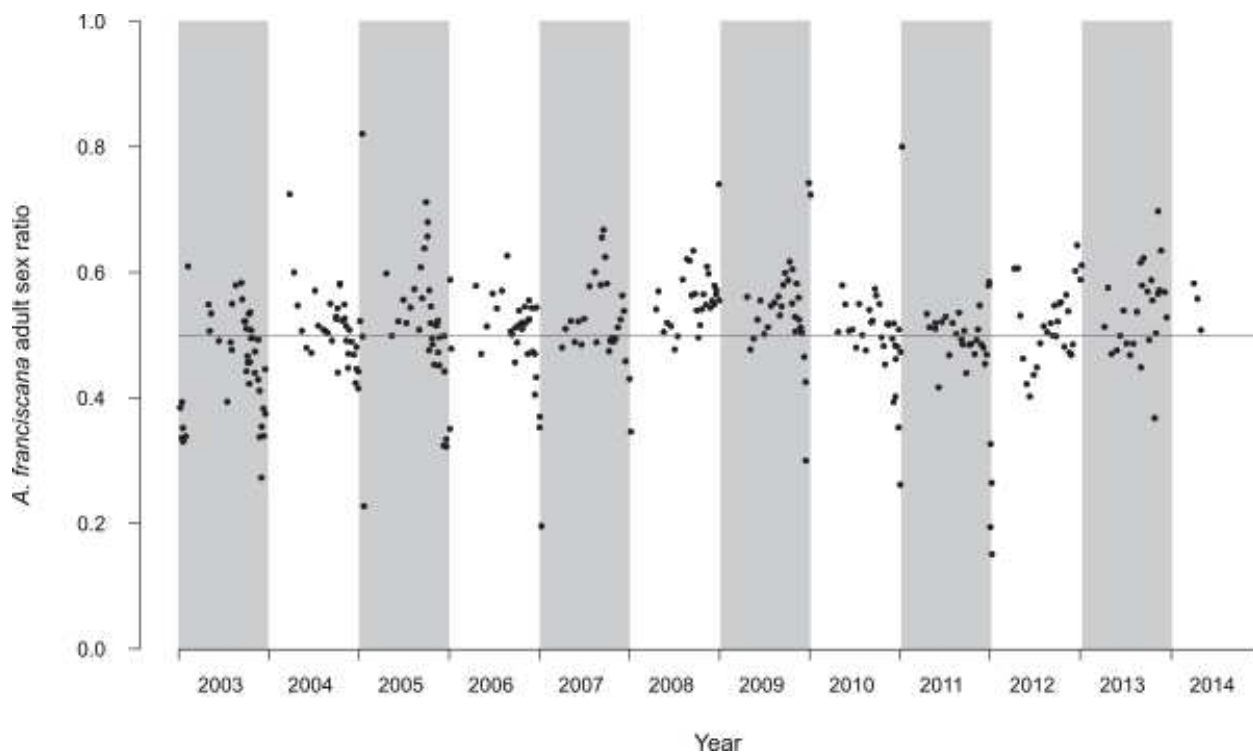


Figure S1, related to Experimental Procedures. Fluctuations in the adult sex ratio in *A. franciscana*'s native range. Data are for the Great Salt Lake, Utah, USA, one of the largest populations of *A. franciscana* in its native range. The gray line represents the equilibrium sex ratio of 0.5.

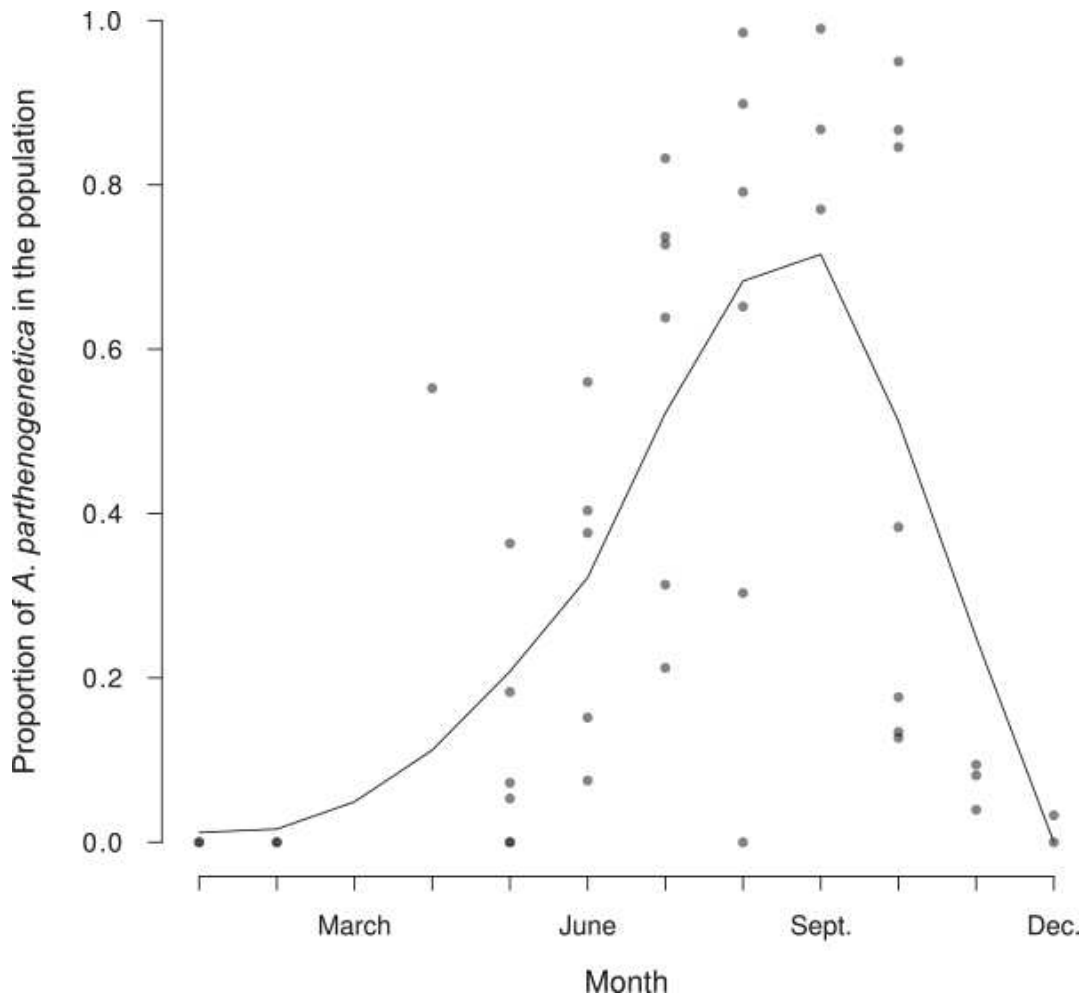


Figure S2, related to Figure 1. *A. parthenogenetica* is seasonally present in Southern France, peaking in summer and absent in winter. This plot includes 44 of the 55 field samples; we excluded data from two isolated sites where *A. franciscana* has completely replaced *A. parthenogenetica* (Fangouse, Site 9 after 2013). Darker points indicate overlapping data; the line represents a 2nd-degree polynomial local regression (LOESS) fitting. The LOESS-predicted value for December was manually corrected from -0.1 to 0.0.

Table S1, related to Figure 1. AIC model comparisons for the invasive range field data. We compared the statistical models explaining the adult sex ratio of *A. franciscana* in our field samples from Southern France using AIC. Fixed factors were: *Proportion parthenogenetics*, *Sampling month* (1 to 12), *Sampling month*², *Saltern* (Aigues-Mortes or Gruissan). Months were rescaled by 1/12 to make computation easier. *Site* was included as a random intercept in all models.

Model	df	AIC	ΔAIC	Fixed factors
1	5	352.8	0.0	Prop. parthenogenetics + Month + Month ²
2	4	353.2	0.4	Prop. parthenogenetics + Month ²
3	6	354.4	1.6	Prop. parthenogenetics + Saltern + Month + Month ²
4	5	354.9	2.1	Prop. parthenogenetics + Saltern + Month ²
5	4	356.2	3.4	Prop. parthenogenetics + Month
6	7	356.4	3.6	Prop. parthenogenetics + Month + Month ² + Saltern + Prop. parthenogenetics : Saltern
7	6	356.9	4.1	Prop. parthenogenetics + Month ² + Saltern + Prop. parthenogenetics : Saltern
8	5	358.1	5.3	Prop. parthenogenetics + Saltern + Month
9	6	360.1	7.2	Prop. parthenogenetics + Month + Saltern + Prop. parthenogenetics : Saltern
10	3	369.8	17.0	Prop. parthenogenetics
11	4	371.8	19.0	Prop. parthenogenetics + Saltern
12	5	373.8	21.0	Prop. parthenogenetics + Saltern + Prop. parthenogenetics : Saltern
13	3	390.9	38.0	Month
14	3	392.5	39.7	Month ²
15	4	392.8	39.9	Month + Month ²
16	3	412.7	59.9	Saltern
17	2	414.8	62.0	1

Table S2, related to Figure 2. Offspring produced by the parental groups over the course of the experiment. Only the adults included in the final dataset are included here. Values in bold are summed across the entire treatment; the mean \pm binomial SEM is also given for the (adult) sex ratio of the offspring. *A. p.* = *A. parthenogenetica*; *A. f.* = *A. franciscana*.

Treatment	Nauplii:	Adults:				
	Total	Total	# <i>A. p.</i> females	# <i>A. f.</i> males	# <i>A. f.</i> females	<i>A. f.</i> sex ratio
Invasive range	2070	446	-	227	219	0.51 \pm 0.03
Replicate 1	290	18	-	11	7	
Replicate 2	270	46	-	22	24	
Replicate 3	300	77	-	31	46	
Replicate 4	170	63	-	29	34	
Replicate 5	120	7	-	2	5	
Replicate 6	160	38	-	23	15	
Replicate 7	210	55	-	27	28	
Replicate 8	180	38	-	24	14	
Replicate 9	180	79	-	47	32	
Replicate 10	190	25	-	11	14	
Invasive range*	1540	230	-	111	119	0.48 \pm 0.04
Replicate 1	840	158	-	75	83	
Replicate 2	700	72	-	36	36	
Invasive range + ♀	6670	667	-	397	270	0.60 \pm 0.02
Replicate 1	890	136	-	75	61	
Replicate 2	640	59	-	30	29	
Replicate 3	760	48	-	34	14	
Replicate 4	640	51	-	33	18	
Replicate 5	730	76	-	43	33	
Replicate 6	340	25	-	12	13	
Replicate 7	690	47	-	26	21	
Replicate 8	380	118	-	79	39	
Replicate 9	630	50	-	26	24	
Replicate 10	970	57	-	39	18	
Invasive range + P	12710	1025	693	206	126	0.62 \pm 0.03
Replicate 1	2190	247	162	55	30	
Replicate 2	980	106	90	9	7	
Replicate 3	1160	94	28	39	27	
Replicate 4	1410	104	56	30	18	
Replicate 5	1440	143	125	10	8	
Replicate 6	740	80	66	10	4	
Replicate 7	1040	59	39	12	8	
Replicate 8	860	35	26	4	5	
Replicate 9	1680	115	68	34	13	
Replicate 10	1210	42	33	3	6	
Native range + P	13990	1451	918	308	225	0.58 \pm 0.02
Replicate 1	890	119	65	29	25	
Replicate 2	1230	43	27	11	5	
Replicate 3	2480	427	274	95	58	
Replicate 4	1100	104	69	26	9	
Replicate 5	1130	91	39	23	29	
Replicate 6	1420	86	40	27	19	
Replicate 7	1160	119	85	24	10	
Replicate 8	1770	80	56	13	11	
Replicate 9	1400	145	90	29	26	
Replicate 10	1410	237	173	31	33	

Table S3, related to Table 2. AIC model comparisons for the experimental data. Based on the experimental results (Table S2), AIC model comparisons via contrast manipulation clearly support the “ancestral trait” scenario explaining *A. franciscana*’s sex ratio skew in the presence of *A. parthenogenetica*.

Model	df	AIC	ΔAIC
Ancestral trait	3	485.8	0.0
Ancestral trait + an effect of absolute number	7	487.5	1.7
Novel trait: indiscriminate trigger	3	489.7	3.9
Null model	2	490.3	4.5
Interference	3	492.9	7.1
Novel trait: discriminate trigger	3	492.9	7.1

Table S4, related to Experimental Procedures. Natural and experimental sex ratios of *Artemia*. Sex ratios are given for various sexual *Artemia* species, as reported in the literature, for *Artemia* that were (1) hatched from diapausing eggs under experimental conditions, or (2) sampled from natural populations. Values preceded by ‘~’ were visually inferred from graphics of the cited references.

Species	Sampling location	Sex ratio (males/all)	Reference
1. Sex ratios under experimental conditions			
<i>Artemia franciscana</i>	Bohai Bay, PR China	0.43-0.48	[S1]
"	Great Salt Lake, USA	~0.5	[S2]
"	Oaxaca, Mexico	~0.5	[S2]
"	San Francisco Bay, USA	~0.5	[S2]
<i>Artemia salina</i>	?	0.49-0.52 when raised on high-quality algae 0.40 when raised on low-quality algae	[S3]
"	Megrine, Tunisia	0.53	[S4]
"	Sabkhet El Adhibet, Tunisia	0.51	[S4]
2. Sex ratios in natural populations			
<i>Artemia franciscana</i>	Great Salt Lake, USA	0.15-0.82	S1 Fig.
"	Great Salt Lake, USA	0.09-0.77	[S5]
"	Great Salt Lake, USA	0.5-0.8	[S6]
"	Carmen Island, Mexico	0.35-0.46	[S7]
"	Sète-Villeroy, France	0.38-0.62	[S8]
"	Villeneuve, France	0.82	[S8]
"	Mesquer, France	0.49	[S9]
"	Guérande, France	0.38-0.63	[S9]
"	Noirmoutier, France	0.51	[S9]
"	St Hilaire de Riez, France	0.57	[S9]
"	Ile d’Oléron, France	0.65	[S9]
<i>Artemia monica</i>	Mono Lake, USA	~0.4-0.8	[S10]
<i>Artemia salina</i>	Chott Marouane, Algeria	0.57-0.63	[S11]
"	Sebkha Ez-Zemoul, Algeria	0.64-0.97	[S12]
<i>Artemia urmiana</i>	Lake Urmia, Iran	0.33-0.69	[S13]

Table S5, related to Experimental Procedures. Numbers of adult male and female *A. franciscana* and adult *A. parthenogenetica* in our field samples from Southern France. ‘Unidentified’ females were those whose species could not be easily determined; for the calculations we assumed that the ratio of unidentified *A. franciscana* to unidentified *A. parthenogenetica* females was the same as that of identified *A. franciscana* to identified *A. parthenogenetica* females.

Sampling date	Saltern	Site	<i>A. franciscana</i>		<i>A. parthenogenetica</i>	Unidentified	Total
			# Males	# Females	# Females	# Females	
07/04/2010	Aigues-Mortes	Site 3	53	23	84	21	181
22/07/2010	Aigues-Mortes	Fangouse	42	36			78
22/07/2010	Aigues-Mortes	Site 1	3	2	14		19
22/07/2010	Aigues-Mortes	Site 3	8	9	30		47
22/07/2010	Aigues-Mortes	Site 9	90	46	61	4	201
22/09/2010	Aigues-Mortes	Caitive Nord	1		98		99
22/09/2010	Aigues-Mortes	Site 4	11	2	85		98
17/11/2010	Aigues-Mortes	Fangouse	58	50			108
19/01/2011	Aigues-Mortes	Fangouse	48	49			97
12/07/2011	Aigues-Mortes	Site 4	33	17	243	11	304
26/07/2011	Aigues-Mortes	Site 4	33	17	125	12	187
09/08/2011	Aigues-Mortes	Site 9	64	44	47		155
07/09/2011	Aigues-Mortes	Fangouse	54	51			105
07/09/2011	Aigues-Mortes	Site 4	18	2	67		87
06/12/2011	Aigues-Mortes	Fangouse	80	49			129
10/01/2012	Aigues-Mortes	Pont de Gazette	52	47		1	100
01/02/2012	Aigues-Mortes	Site 4	21	12		5	38
01/08/2012	Aigues-Mortes	Puits Romains	20		45	132	197
01/08/2012	Aigues-Mortes	Site 3	1		66		67
01/08/2012	Aigues-Mortes	Site 4	23	6	105	5	139
28/11/2012	Aigues-Mortes	Site 4	55	19	6	6	86
13/02/2013	Aigues-Mortes	Site 9	32	40			72
26/02/2013	Aigues-Mortes	Fangouse	25	20		1	46
23/04/2013	Aigues-Mortes	Fangouse	39	37			76
31/05/2013	Aigues-Mortes	Pont de Gazette	56	33	5		94
31/05/2013	Aigues-Mortes	Puits Romains	37	43			80
31/05/2013	Aigues-Mortes	Site 4	72	54	26	12	164
26/06/2013	Aigues-Mortes	Pont de Gazette	22	15	3		40
16/10/2013	Aigues-Mortes	Caitive Sud	2	2	75	21	100
16/10/2013	Aigues-Mortes	Pont de Gazette	66	12	12	12	102
16/10/2013	Aigues-Mortes	Puits Romains	35	19	8	1	63
16/10/2013	Aigues-Mortes	Site 4	16	2	117		135
22/10/2013	Aigues-Mortes	St. Louis	42	29	11		82
23/10/2013	Gruissan	PM 2	140	69	121	22	352
23/10/2013	Gruissan	Station 2	34	10	234	14	292
15/11/2013	Aigues-Mortes	Pont de Gazette	33	14	5	1	53
15/11/2013	Aigues-Mortes	Puits Romains	46	50	4	1	101
03/12/2013	Aigues-Mortes	Site 9	248	164	14	2	428
17/12/2013	Aigues-Mortes	Site 9	117	80		1	198
07/01/2014	Aigues-Mortes	Site 9	162	92		3	257
21/01/2014	Aigues-Mortes	St. Louis	125	103		3	231
04/02/2014	Aigues-Mortes	Site 9	103	114			217
18/02/2014	Aigues-Mortes	Site 9	90	108		1	199
23/05/2014	Aigues-Mortes	Caitive Nord	92	73	13	2	180

23/05/2014	Aigues-Mortes	Pont de Gazette	66	61		1	128
23/05/2014	Aigues-Mortes	Puits Romains	50	54	59	2	165
24/06/2014	Aigues-Mortes	Pont de Gazette	63	59	21	2	145
08/07/2014	Aigues-Mortes	Pont de Gazette	30	21	14	1	66
01/08/2014	Aigues-Mortes	Pont de Gazette	51	17	115	18	201
12/08/2014	Aigues-Mortes	Pont de Gazette	63	32			95
30/09/2014	Aigues-Mortes	Site 9	22	21			43
16/06/2015	Aigues-Mortes	Caitive Nord	21	10	35	9	75
16/06/2015	Aigues-Mortes	Pont de Gazette	45	11	30	7	93
16/06/2015	Aigues-Mortes	Site 4	51	12	38	8	109
16/06/2015	Aigues-Mortes	Site 9	30	22			52

Supplemental Experimental Procedures

Field observations of *A. franciscana* sex ratios in the invasive range

Statistical analysis

We analyzed the field sex ratio data using generalized linear mixed-effects models with a binomial error distribution [package lme4 in R 3.1.0; S14, S15]. Because *Artemia* in a saltern can be temporarily segregated into different basins, which we call sites, all our models included *Site* as a random effect. The full model had the following fixed effects: *Proportion parthenogenetics* (the proportion of *A. parthenogenetica* present in the sample), *Sampling month*, *Sampling month*² (which allowed for quadratic variation in sex ratio during the year), *Saltern* (Aigues-Mortes or Gruissan), and an interaction between *Saltern* and *Proportion parthenogenetics* (allowing *A. franciscana* from different salterns to have different responses to the presence of *A. parthenogenetica*). We selected the best combination of these fixed factors using the Akaike information criterion [S16].

Experimental test of sex ratio-biasing in *A. franciscana*

Experimental conditions

To produce our experimental animals, we hatched native-range *A. franciscana* from diapausing eggs sampled in the Great Salt Lake, USA (41°10' N, 112°35' W; 2007), and invasive-range *A. franciscana* and *A. parthenogenetica* from cysts sampled in Aigues-Mortes, France (43°34' N, 4°11' E; 2013). All cysts were stored in dry condition at 4°C; they were hatched following a protocol modified from Bengtson et al. [S17]. Cysts were rehydrated with deionized water for 2 hours, decapsulated by a brief exposure (< 10 min) to 2% sodium hypochlorite, and rinsed with fresh water. Decapsulated cysts were incubated until emergence in an aerated saline medium (salinity 5 g/L) at 28°C and under constant light. We produced the saline medium by mixing concentrated brine (Camargue Pêche, France) with deionized tap water. All brine was autoclaved before use to ensure it contained no horizontally transmitted parasites; there are no (known) vertically transmitted parasites in these populations, so individuals hatched from dormant eggs are in principle parasite-free. After emergence, the experimental *Artemia* were transferred to large tanks of non-aerated medium at 23°C under natural light, and salinity was gradually increased to 90 g/L. They were fed *ad libitum* with a mixed solution of two parts *Tetraselmis chuii* (6.8×10^9 cells/L, Fitoplancton marino, Spain) to one part powdered yeast solution (0.4 g/L, Gayelord Hauser, France). Shortly before sexual maturity, we separated the individuals by species and sex [S18] and formed the parental groups (see above). All *A. franciscana* females used in the experiment were virgins.

Parental groups were kept at a constant density (40 individuals/L), light (36 W), temperature (21°C) and salinity (90 g/L). They were fed a standardized volume of *T. chuii*/yeast solution daily (5 mL or 20 mL for groups of 10 or 40 individuals, respectively; solution concentration: 3.4×10^9 *T. chuii* cells + 0.2 g yeast/L), and their mortality was monitored. The nauplii harvested from the parental groups were reared in separate jars under the same temperature, light and salinity conditions as the parental groups; their density was fixed (200 nauplii/liter, based on the initial count) and they were fed a standardized volume of the *T. chuii*/yeast solution daily (0.1 mL/nauplius, based on the initial count).

Statistical analysis

We analyzed the offspring sex ratios produced in the experiment using generalized linear mixed-effects models with a binomial error distribution [package lme4 in R 3.1.0; S14, S15]. The response variable was the number of *A. franciscana* males (successes) and females (failures) counted per parental group and per clutch. All models included *Parental group* as a random effect and *Treatment* as a fixed effect. We tested the scenarios described above by contrast manipulation, forcing the factor *Treatment* to have only two levels and attributing these levels to the different treatments (see Table 2). We then selected the best model by AIC comparison [S16]. The effect of absolute number of parents was tested by taking the best model and attributing a third factor level to treatment “invasive range*”. We also tested models that took parental mortality and time of collection into account, but these did not improve the AIC score and were discarded.

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CHAPTER 2

DISCRIMINATION AGAINST HETEROSPECIFIC AND NON-RECEPTIVE FEMALES DURING PRE-COPULATORY MATE GUARDING IN *ARTEMIA*

Eva J. P. Lievens, Thomas Lenormand

Pre-copulatory mate guarding is a reproductive strategy in which males secure a mating opportunity by guarding females that are not yet receptive. Guarding is a costly investment, so males should prefer to guard conspecific females that are close to receptivity. We studied the mate guarding behavior of *Artemia franciscana*, a bisexual crustacean species, in its invasive range in Southern France. We sampled guarding pairs and random individuals from three field sites, one of which also contained the native asexual species *Artemia parthenogenetica*. At all sites, amplexing pairs were more likely to contain females that were close to receptivity, although the pattern was not entirely predictable across reproductive stages. Where they were present, *A. parthenogenetica* females were strongly discriminated against. We also confirm previous reports of size-assortative pairing in *Artemia*. Our findings support the theoretical prediction that males should prefer females that maximize the return on their guarding investment, and provide the first evidence for species-based discrimination in *Artemia*. We discuss our results in terms of the emerging consensus on male mate choice in *Artemia*.

In preparation.

INTRODUCTION

Mate guarding is a common male competitive strategy, employed to increase the chances of fertilizing a given female's eggs (Parker 1974). Pre-copulatory mate guarding, in which males guard an as-yet-unreceptive female, is believed to evolve when female receptivity is time-limited (Parker 1974, Yamamura 1987). This behavior has large rewards for the male (the certainty of fertilization), but it comes with an intrinsic opportunity cost (he cannot fertilize other females while he is guarding), and can have energetic and survival costs for both partners (reviewed by Jormalainen 1998).

Because pre-copulatory mate guarding is a competitive and costly investment, its duration should be optimized (Parker 1974): theoretical analyses suggest that the time spent guarding should increase as guarding becomes less costly and sexual partners scarce (Grafen and Ridley 1983, Yamamura 1987, Yamamura and Jormalainen 1996).

Many of the empirical results supporting pre-copulatory guarding theory and its assumptions come from studies of sexual behavior in crustaceans. This group has provided evidence for the critical assumptions that males can judge a female's reproductive stage (e.g. Thompson and Manning 1981, Borowsky and Borowsky 1987, Weeks and Benvenuto 2008) and the likelihood of encountering females (e.g. Dunham and Hurshman 1990). Furthermore, several cases support the prediction that the guarding criterion should be earlier in populations with high male densities or male-biased sex ratios (e.g. Manning 1980, Iribarne et al. 1995, Dick and Elwood 1996). Finally, there is some evidence that males weigh the guarding criterion by the expected pay-off of the mating: female size is correlated with fecundity in many crustaceans, and males appear to prefer larger females (e.g. Elwood et al. 1987, Reading and Backwell 2007).

One interesting crustacean model to study the evolution of pre-copulatory mate guarding is the genus *Artemia*, also called brine shrimp (Crustacea: Branchiopoda: Anostraca). Males of sexual *Artemia* species invest heavily into pre-copulatory mate guarding, called 'amplexus'. Amplexing pairs form when the male grasps the female, using a pair of modified antennae (claspers, Wolfe 1973). The pair then swims in tandem until copulation occurs, and separate shortly afterwards. Female *Artemia* produce a clutch every four to six days (Bowen 1962, Metalli and Ballardin 1970). Amplexus can last as long as three days (Lent 1971, Wolfe 1973), so guarding can begin over halfway through the reproductive cycle. This lengthy amplexus should impose intense selection on males to optimize their investment (Edward and Chapman 2011).

Previous work has demonstrated that amplexing pairs of *Artemia* show size- and population-assortative mating (Browne et al. 1991, Forbes et al. 1992), and there is evidence that the probability of amplexus varies according to the female's reproductive stage when one pair is followed over time (Pastorino et al. 2002). This may be due to male choice or female choice:

Artemia females can attempt to reject amplexus (Forbes et al. 1992, Tapia et al. 2015), or choose not to open their gonopore for copulation (Belk 1991, Rogers 2002).

In this study, we investigated whether amplexing *Artemia* discriminate against unreceptive or heterospecific females in natural populations. We sampled random individuals and amplexing pairs from a French *Artemia* population containing the asexual species *Artemia parthenogenetica* and the sexual species *Artemia franciscana*. The latter was introduced into the area in 1970, from a native range lacking sympatric congeners (Amat et al. 2005, Rode et al. 2013c). We demonstrate that amplexing pairs are size-assorted and more likely to contain close-to-receptive females, and that *A. parthenogenetica* are strongly discriminated against.

METHODS

Data collection

We sampled *Artemia* in three different basins (Fangouse, Puit Romain, and Site 9) of the saltern of Aigues-Mortes, France (43.53°N, 4.21°E), on October 19th, 2015. The goal of our sampling was to obtain a representative sample of the *Artemia* population at each site, and a separate sample of amplexing pairs. At each site, we first collected a large quantity of *Artemia* from the population using a fishing net. From this, a random subset of several hundred individuals (including amplexing pairs) was removed, sacrificed and stored in a 50% ethanol solution. This is the ‘random sample’ hereafter. From the remaining *Artemia*, we isolated 96 amplexing pairs, which were sacrificed and stored individually, forming the ‘amplexing sample’.

In the laboratory, we phenotyped the collected individuals. We noted species, sex, body length (to the nearest 0.5 mm) for all individuals. We also identified each female’s reproductive stage. This can be done externally because *Artemia* are translucent, a state maintained by the 50% ethanol solution our samples were stored in. We classified the reproductive stages following Metalli & Ballardin (1970) (see Fig. 1). In stage A, nothing is visible. Because it could not be distinguished from sexual immaturity, we did not score this stage. During stage B, oocytes begin to accumulate yolk granules in the ovaries, and are visible as opaque dots in the first abdominal segments. The oocytes reach diakinesis in stage C, which is macroscopically indistinguishable from stage B. We thus scored any females with an empty ovisac and full ovaries as belonging to stage C. The female then molts and ovulates, pushing the oocytes into the lateral oviducts (stage D). Copulation occurs in this stage. The oocytes are then pushed into the ovisac, where they are fertilized (Criel and Macrae 2002) and mature into dormant eggs or live young (stage E). *Artemia* have overlapping egg cycles: soon after the first brood enters stage E, a second brood will start to mature in the ovaries. We thus distinguish between stages E-A (full ovisac, empty ovaries) and E-B (full ovisac, full ovaries). The whole cycle takes 4-6 days in *A. franciscana*, with stages E-A and E-B both taking several days and stages C and D measured in hours (Bowen 1962). We expect mate guarding to be important in the lead-up to

stage D, when females are receptive, but be counter-selected afterwards (stage E-A and onwards).

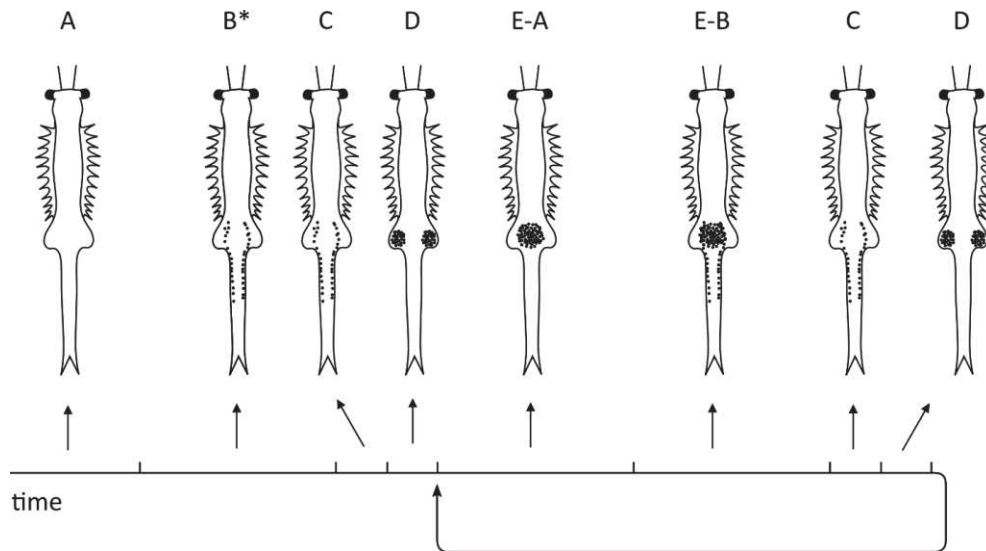


Figure 1. Reproductive stages of *Artemia*, as described by Metalli & Ballard (1970) for external identification. A female's first brood starts at the left; towards the end of the first brood, a second brood starts maturing in the ovaries (stage E-B: the first brood is in stage E, the second brood is in stage B). All following broods overlap in this manner. Copulation occurs during stage D. The timeline presents a rough guide to the duration of each stage. *Scoring issues: for the first brood, stage B is indistinguishable from stage C; the former was not scored and the latter was scored as C.

At one site, we noticed the presence of the cestode *Flamingolepis liguloides*, a flamingo parasite that uses *Artemia* as an intermediate host, and has castrating effects on *A. parthenogenetica* (Amat et al. 1991a, Sánchez et al. 2012) and to a lesser extent *A. franciscana* (Redón et al. 2015a). For these samples, therefore, we also scored infection by *F. liguloides*. Some females at this site were of adult size but had undeveloped reproductive systems, and others had developed reproductive systems that did not contain any oocytes or embryos. Both of these traits are typical of castration by *F. liguloides*. However, not all of the aforementioned females contained visible cestodes, so we classified their reproductive stage as 'empty' instead of 'castrated'.

Statistical analyses

First, we investigated whether the likelihood that a female was part of an amplexing pair depended on her reproductive stage, species, or infection status. To analyze the effects of reproductive stage, we used a generalized linear model with *Amplexus* as a binary response variable. We included *Reproductive stage* and *Site* as possible fixed effects, and compared models using the corrected AIC (Hurvich and Tsai 1989). To avoid confounding species effects, the handful of amplexed *A. parthenogenetica* females were excluded from this analysis. To study the effects of female species, we restricted our dataset to females sampled in Puit Romain (where *A. parthenogenetica* was present, see Results). We used a similar model, substituting

Species for Site as a possible fixed effect. Finally, we analyzed the effects of infection with *F. liguloides* on the probability of amplexus using Fisher's exact tests, restricted to females from Site 9 (where *F. liguloides* was present, see Results). Because infection did not correspond completely to reproductive 'emptiness', we tested the two factors separately.

Second, we looked for assortative mating by size at the different sites. For each site, we tested for correlations between male and female length using Pearson's product moment. The slope of significant correlations was calculated using a linear regression with standardized length data for each sex (standardization avoids implying causality in the regression).

All analyses were done in R (R Core Team 2015); linear modeling was done using the package lme4 (Bates et al. 2015).

RESULTS

We collected *Artemia* at the sites Fangouse, Puit Romain, and Site 9 (counts provided in Table 1, proportions in Table 1). *A. parthenogenetica* were only present at the site Puit Romain, where they made up 11% of the total population, 26% of the females, and 6% of the amplexed females. 3% of the individuals in Site 9 were infected with *F. liguloides*. This included eight females, all of whom had undeveloped (n = 2) or developed-but-empty (n = 6) reproductive systems. Two further females in Site 9 had developed-but-empty reproductive systems but were not visibly infected by *F. liguloides* (they may have been infected by developing larvae or by other cestode species, Sánchez et al. 2012).

Table 1. Proportional data from the various samples: sex ratio of the population, proportion of *A. parthenogenetica* in the population, and the distribution of reproductive stages among *A. franciscana* females. Empty cells indicate the proportion is not applicable; *A. f.*, *A. franciscana*; *A. p.*, *A. parthenogenetica*.

Sample	Sex ratio of the population	Prop. of <i>A. p.</i> in the population	Prop. of <i>A. f.</i> females in the reproductive stages				
			empty	C	D	E-A	E-B
Fangouse							
Random	0.49	0.00	0.12	0.04	0.34	0.50	
Amplexing			0.10	0.07	0.10	0.72	
Puit Romain (<i>A. f.</i>)							
Random	0.64 ¹ / 0.57 ²	0.11	0.24	0.01	0.37	0.38	
Amplexing			0.16	0.04	0.00	0.74	
Site 9							
Random	0.56	0.00	0.04	0.09	0.01	0.53	0.33
Amplexing			0.04	0.11	0.09	0.03	0.72

Notes: ¹ Counting only *A. franciscana* individuals, i.e. (# males)/(# *A. f.*). ² In the whole population, i.e. (# males)/(# *A. f.* + # *A. p.*).

A. franciscana size was variable across sexes and sites (Fig. 2). Females were larger than males in all samples. At the sites Fangouse and Site 9, amplexing individuals (males and females) were smaller than randomly sampled individuals; this was not the case for Puit Romain. *A. parthenogenetica* females, sampled from Puit Romain, were slightly larger than *A. franciscana*

Table 1. Numbers of *A. franciscana* males, *A. franciscana* females, and *A. parthenogenetica* females counted in each sample. Provided are the total counts and the division of females into reproductive stages. Empty cells indicate the count does not apply; *A. f.*, *A. franciscana*; *A. p.*, *A. parthenogenetica*.

Sample	Total males	Total females		Reproductive stages of <i>A. f.</i> females					Reproductive stages of <i>A. p.</i> females			
	<i>A. f.</i>	<i>A. f.</i>	<i>A. p.</i>	empty	C	D	E-A	E-B	C	D	E-A	E-B
Amplexing samples												
Fangouse	96	96	0		10	7	10	69				
Puit Romain	96	90	6		15	4	0	71	1	0	1	4
Site 9	96	96	0	4 ¹	11	9	3	69				
Random samples												
Fangouse	104	100	0		12	4	34	50				
Puit Romain	174	98	34		24	1	36	37	6	0	16	12
Site 9	115 ³	89	0	4 ²	8	1	47	29				

Notes: Recorded *F. liguloides* prevalences were: ¹ 2/4, ² 3/4, ³ 1/115.

females (ranges: 6.0 to 8.5 mm in the amplexing sample; 6.5 to 9.0 mm in the random sample, with most falling between 6.5 and 8.0 mm).

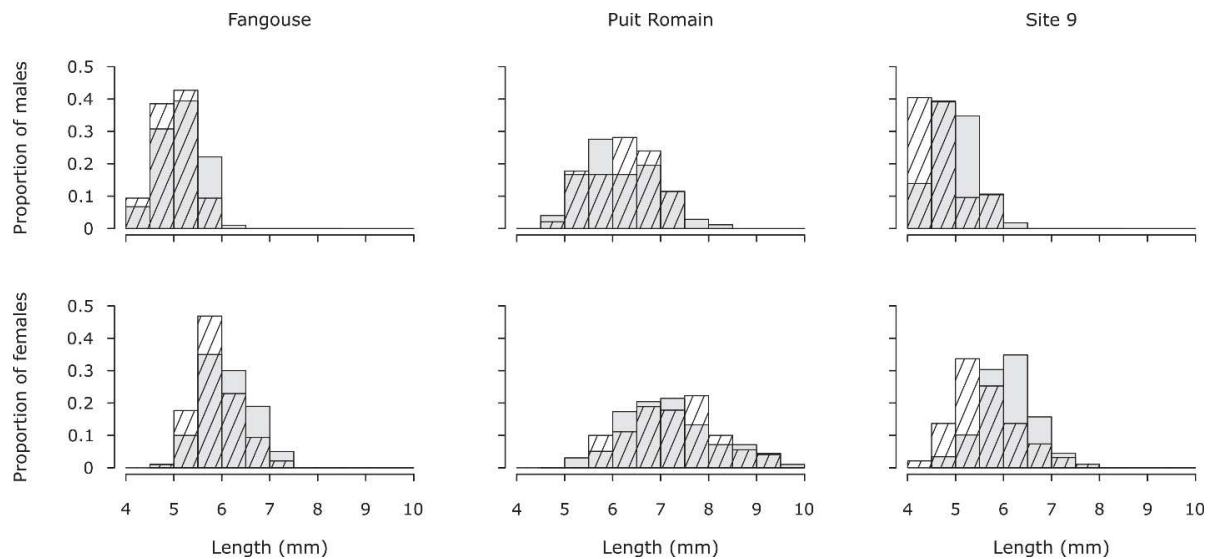


Figure 2. Size distributions of *A. franciscana* males (top) and females (bottom), in the random samples (gray bars) and amplexing samples (hatched bars).

Amplexed females differed from random females in reproductive stage and species, but not in infection status. For *A. franciscana* females, amplexus was much less likely in stage E-A, more likely in stage E-B, seemingly random in stage C, and much more likely in stage D (Fig. 3). This ranking was very robust, although the precise relationship between amplexus and reproductive stage was dependent on the sampling site ($\Delta\text{AICc} \geq 6.0$, Supp. Table 1). *A. parthenogenetica* females were 5 times less likely to be part of an amplexing pair in Puit Romain ($\Delta\text{AICc} \geq 7.3$, Supp. Table 1), with some indications that the effect of reproductive stage was also weaker in this species ($\Delta\text{AICc} = 0.6$, Supp. Table 1). The likelihood of amplexus for *A. parthenogenetica* females was independent of size (data not shown). Lastly, the likelihood of amplexus was independent of cestode infection (odds ratio = 0.6, $p = 0.67$) and reproductive status (odds ratio = 0.9, $p = 1.00$) for Site 9 females.

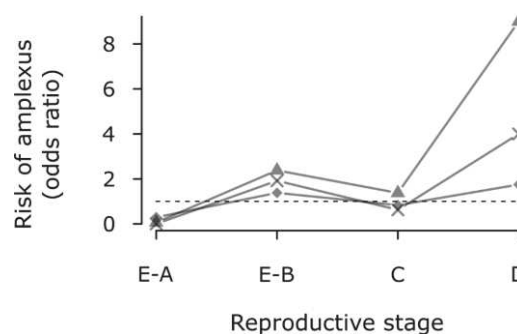


Figure 3. Relative risk of amplexus, as a function of the female's reproductive stage. Reproductive stages are ordered from the furthest moment from female receptivity (left) to the moment of female receptivity (right). Symbols show the values for each sample (diamond, Fangouse; cross, Puit Romain; triangle, Site 9). The

dashed line marks an odds ratio of 1 (equally likely to be in amplexus as not). Only *A. franciscana* individuals were included in these analyses.

Amplexing pairs of *A. franciscana* were size-assorted at the sites Puit Romain ($t = 4.7$, $p < 0.0001$) and Site 9 ($t = 2.4$, $p = 0.02$), but not at Fangouse ($t = 0.2$, $p = 0.85$) (Fig. 4).

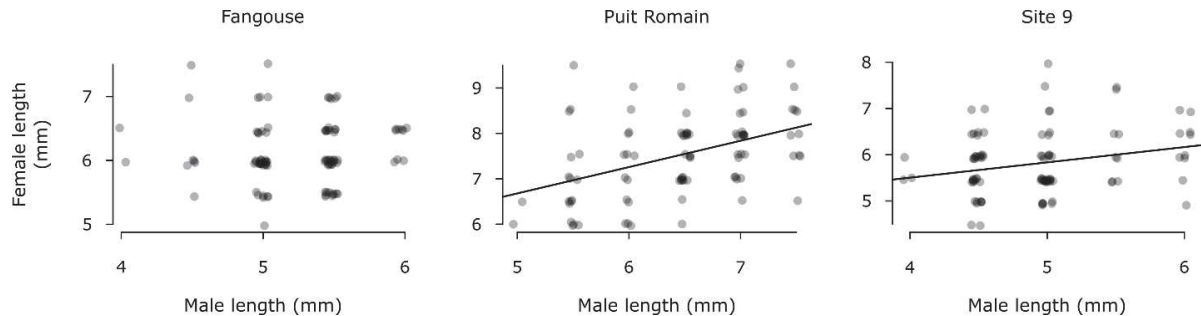


Figure 4. Size of males and females in amplexing pairs, with significant correlations shown (Puit Romain: $y = 0.45x$, $R^2 = 0.19$, $p < 0.0001$; Site 9: $y = -0.02 + 0.24x$; $R^2 = 0.05$; $p = 0.02$). Individuals were measured to the nearest 0.5 mm, but points are jittered for clarity. Overlapping points shade to black. Only *A. franciscana* pairs were included in these analyses.

DISCUSSION

We studied discrimination during mate guarding in *A. franciscana*, finding that amplexus was more likely when females were conspecific and close to sexual receptivity, and that amplexing pairs were size-assorted. We compare these results with previous information on mate choice in *Artemia*, and describe the emerging consensus.

Duration of female reproductive stages

When females are sampled randomly in a population, the proportion of females in a certain reproductive stage can also be interpreted as the relative duration of that stage. In accordance with previous reports, we found that the stages E-A and E-B were much longer than stages C and D in *A. franciscana* females (Table 2) (Bowen 1962, Metalli and Ballardin 1970). There were some differences between samples, most notably the extended stage E-A at Site 9 and the extended stage C at Puit Romain. Our data did not allow us to see if the longer E-A stage at Site 9 extended the reproductive cycle, or if it was prolonged at the expense of another stage. The extended stage C at Puit Romain may have been a sampling artefact: many of the amplexing females from Puit Romain aborted after sampling, passing from stage E-B to C artificially (this did not occur in the other amplexing samples). This was detectable for amplexing females because they were stored separately, but would not have been for randomly sampled females. If the latter also aborted broods, we may have overestimated the proportion in stage C.

Discrimination by female reproductive stage, species, and size

A. franciscana females were more likely to be part of an amplexing pair when they were close to sexual receptivity, which occurs in stage D (Fig. 3) (cf. Pastorino et al. 2002). The relative risk of amplexus dropped sharply immediately afterwards (stage E-A), then increased as the next batch of oocytes began to mature (stage E-B). The high probability of amplexus during stage E-B, which lasts several days, fits the ‘hours to days’ duration of amplexus reported in the literature (Lent 1971, Wolfe 1973, Belk 1991, Rogers 2002). Oddly, stage C saw a dip in amplexus probability, relative to stage E-B, in all samples (Fig. 3). It is unclear why this should be, since females in this stage are mere hours away from becoming receptive (in stage D). One possibility, alluded to by Browne & Halanych (1989), is that males become dislodged as females give birth to their previous clutch (between stages E-B and C), or during the ensuing molt (during stage C). If so, dislodged males risk the loss of their invested effort if they cannot maintain their claim on the female in some other way during this period.

In addition, females of the asexual species *A. parthenogenetica* were much less likely to be amplexed. Previous experiments in our lab indicate that *A. parthenogenetica* females are tolerant of amplexus (T. Lenormand, unpublished data), suggesting that *A. franciscana* males are able to discriminate between con- and heterospecific females. This is the first report of species discrimination in *Artemia*, and it raises an intriguing question. The sexual species *A. franciscana* was introduced to France in 1970 (Rode et al. 2013c), from a native range in which there are no sympatric congeners (although *A. franciscana* does amplex other sympatric Anostraca occasionally, Belk and Serpa 1992). Has its ability to discriminate between conspecific and asexual females evolved since its invasion? Future work should provide an answer.

A. franciscana pairs were also size-assortative in two of the three samples (Fig. 4), confirming previous results (Forbes et al. 1992). The absence of size assortment in Fangouse may have been caused by the limited variation in body size at that site.

Finally, despite the wealth of discrimination demonstrated by *A. franciscana*, they remained in amplexus when the female partner was castrated by the cestode *F. liguloides*. This is obviously counterintuitive, but it may be explained by the very recent emergence of the parasite in this host: until 2013, *F. liguloides* was never seen infecting *A. franciscana* in Aigues-Mortes (Sánchez et al. 2012, Rode et al. 2013b, personal observation). The selection to discriminate against females castrated by this parasite must therefore be very recent; given time, *A. franciscana* males should evolve to reject these females (e.g. Bollache et al. 2002). It is unknown whether any of the cestodes infecting *A. franciscana* in its native range castrate female hosts (Redón et al. 2015b); if so, it would be particularly interesting to test whether *A. franciscana* males could identify and reject such females.

Male mate choice in Artemia: clasp first, think later

Our results fit well with previous authors' conclusions about male mate choice in *Artemia*, which appears to be a 'clasp first, think later' affair. In experimental settings, male *Artemia* typically attempt amplexus with any female they encounter, without discriminating by female size or species (Belk and Serpa 1992, Forbes et al. 1992, Rogers 2002, T. Lenormand unpublished data). Indeed, Rogers (2002) reports that "male *Artemia franciscana* [...] were also willing to amplex bits of grass shaped like females, and the tip of a pencil". Clearly, *Artemia* males are indiscriminate when amplexus is first attempted and established. When *Artemia* pairs are sampled from a stable population, however, they do show size- and genotype-assortative mating (this study, Browne et al. 1991, Forbes et al. 1992). A striking example of this distinction was given by Forbes et al. (1992), who showed that in experimental tanks, pairs sampled soon after they were formed were not size-assorted, while pairs sampled one week later were. Thus, mate choice emerges if individuals are given time to assess, and possibly separate from, their amplexed partner. In principle, pairs can separate due to male choice (releasing the female) or female choice (dislodging the male). However, females seem to primarily reject males in the first moments of amplexus (Forbes et al. 1992, Rogers 2002, Tapia et al. 2015, pers. obs.), so it is probable that any difference in discrimination between recently-amplexing and established pairs is primarily due to male choice.

Clasping first and thinking later could be an optimized mate selection strategy for male *Artemia*. *Artemia* mate by scramble-competition polygyny (Alcock 1980), in which males avoid aggressive encounters and concentrate their reproductive energy into finding and fertilizing receptive females (Belk 1991). Such a strategy would favor an opportunistic approach to amplexus, with little mate evaluation beforehand (Belk 1991). However, *Artemia* males also invest heavily into mate guarding, and this high level of male investment should favor a considerable capacity for male mate choice (Edward and Chapman 2011). An ability to assess amplexed females, therefore, would be strongly selected for (Forbes et al. 1992). Indeed, similar behaviors have been reported for other crustaceans with reproductive scramble competition (Hunte et al. 1985, Galipaud et al. 2015), suggesting that indiscriminate amplexus coupled with post-amplexus choice is a robust male strategy in these conditions.

Assessment of amplexed females and the overall female population

How do males assess a female's species and reproductive stage? As discussed above, male *A. franciscana* judge their partner's quality during amplexus, ruling out the soluble 'distance' pheromones produced by many aquatic crustaceans for these purposes (e.g. Lonsdale et al. 1998, Zhang et al. 2010). Instead, *Artemia* males may recognize 'contact' pheromones bound to the female's exoskeleton. These are used by many crustacean species (e.g. Thompson and Manning 1981, Borowsky and Borowsky 1987, Lonsdale et al. 1998, Ting et al. 2000, Goetze and Kiørboe 2008, Weeks and Benvenuto 2008), including cladocerans, some of *Artemia*'s closer relatives within the Crustacea (Van Damme and Dumont 2006, La et al. 2014). Alternatively,

males may judge a female's reproductive stage based on morphological cues, or on the intensity of her resistance to amplexus.

To decide whether to release an amplexed female, however, males must also know how she compares to other females in the population (Jormalainen 1998). Forbes et al. (1992) speculated that *A. franciscana* males continue to assess the availability of other females while in amplexus, releasing their amplexed partner if she is of comparatively low quality. A recent study of the amphipod *Gammarus pulex*, however, has shown that males do not base the decision to continue amplexus on the relative quality of their partner (compared to a passing female), but rather on her absolute quality (Galipaud et al. 2015). The authors attribute the lack of comparative decision making – which would be optimal – to the male *Gammarus*' inability to assess female quality at a distance. Instead, through repeated contact with many females, they may judge the overall quality and availability of the female population, and use this information to establish an absolute threshold for amplexus continuation. Given that *Artemia* males also need contact to evaluate females, we favor this mechanism for *A. franciscana*.

CONCLUSION AND PERSPECTIVES

In conclusion, we provide a clear demonstration that *A. franciscana*, as predicted by theory, adjust their mate guarding behavior based on the female's proximity to sexual receptivity. We also show that heterospecific pairs are discriminated against, and speculate that this may be a newly-evolved ability in the invasive *A. franciscana*. In general, our results support the emerging 'clasp first, think later' paradigm of *Artemia* mate choice.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Model selection results for analyses of discrimination based on reproductive stage or species. Abbreviations: *A. f.*, *A. franciscana*; *A. p.*, *A. parthenogenetica*; *w*, Akaike weight.

Analysis	AICc	ΔAICc	w
Amplexus in <i>A. f.</i> females			
~ Reproductive stage * Site	648.3	0	0.93
~ Reproductive stage	654.3	6.0	0.05
~ Reproductive stage + Site	655.8	7.5	0.02
~ Site	783.1	134.8	0.00
Amplexus in Puit Romain females			
~ Reproductive stage * Species	241.7	0	0.57
~ Reproductive stage + Species	242.3	0.6	0.42
~ Reproductive stage	249.0	7.3	0.01
~ Species	298.2	56.5	0.00

DISCUSSION & PERSPECTIVES

Coexisting with *A. parthenogenetica* clearly interferes with the reproductive behavior of invasive *A. franciscana*, but not with every behavior. With regards to sex allocation, reproductive interference was strong: *A. franciscana* females could not distinguish between con- and heterospecifics when judging the population sex ratio, and made faulty sex allocation decisions as a result. For this trait, *A. parthenogenetica* clearly acts as an ‘evolutionary trap’ for the invasive congener (Schlaepfer et al. 2005), and one from which *A. franciscana* has failed to escape. In contrast, there appeared to be little or no interference in mate guarding. As yet, it is unclear whether the ability of *A. franciscana* males to discriminate between con- and heterospecific amplexed partners is a pre-adaptation or a post-invasion adaptation.

Evolution of discrimination against hetero-specific females

Currently, an experiment is underway to test whether *A. franciscana* males from populations in the native range are pre-adapted to discriminate against *A. parthenogenetica* females when mate guarding. If not, we will have demonstrated that the ability to do so evolved after *A. franciscana*’s invasion of Southern France. Such a result would suggest that the mechanisms used by males to evaluate an amplexed female are more flexible, evolutionarily speaking, than those used by females to estimate the population sex ratio.

Sex-specific estimation of population parameters

The *Artemia* system in Aigues-Mortes may provide a unique opportunity to study the way males and females perceive and react to population cues. In Chapter 1, we showed that female *A. franciscana* can judge the sex ratio of a population. Based on the results of Chapter 2, we speculate that *A. franciscana* males may have a similar ability. Mate guarding theory expects an ability to judge the receptivity of individual females (which *A. franciscana* males have) to be coupled with an ability to assess the overall availability and receptivity of females in the population (Jormalainen 1998), so that males may adjust their willingness to guard non-receptive females as a function of the population sex ratio. This has been confirmed for various crustacean species (e.g. Manning 1980, Iribarne et al. 1995, Dick and Elwood 1996, Benvenuto et al. 2009), and we therefore expect *A. franciscana* males to have this ability as well.

Experiments testing whether *A. franciscana* males adjust their guarding criteria based on the overall sex ratio of the population could have three potential outcomes. First, if males can assess the population sex ratio without recognizing asexuals (like *A. franciscana* females), we could conclude that the two *A. franciscana* sexes are likely to use the same population cues. This would be the most parsimonious use of signaling in the species. Second, if males can assess the population sex ratio correctly (distinguishing between conspecifics and asexuals), we could conclude that *A. franciscana* males and females use a separate set of cues to evaluate the population (e.g. accumulated information from repeated contacts, Galipaud et al. 2015, vs.

distance pheromones, Tapia et al. 2015). Finally, if *A. franciscana* males are unable to assess population sex ratio at all, we may wonder why only one of the sexes is thus constrained.

Amplexus in the time of F. liguloides

One particularly interesting result of Chapter 2 is that *A. franciscana* males do not discriminate against *F. liguloides*-infected, castrated females when mate guarding. Until 2013, *F. liguloides* were never recorded infecting *A. franciscana* in Aigues-Mortes, so it is not unreasonable that males sampled in 2015 were unable to detect infections with this cestode (Sánchez et al. 2012, Rode et al. 2013b). Now, however, evidence indicates that *F. liguloides* is colonizing *A. franciscana* at a rapid pace (see Appendix 2), so selection to discriminate against castrated females should be increasing equally rapidly (Bollache et al. 2002). In the future, it would be particularly interesting to use resurrection studies (reviving dormant *Artemia* cysts of different ages, cf. Rode et al. 2011) to investigate whether males evolve this ability as *F. liguloides* establishes itself in their population.

SECTION 2:

PARASITE SPECIALIZATION

OVERVIEW

Context

Parasites range from extremely host-specific to widely generalist, with the majority of parasites infecting multiple host species (e.g. Cleaveland et al. 2001, Taylor et al. 2001, Streicker et al. 2013). The degree of specialization is generally considered to be an evolved trait. Indeed, phylogenetic studies have shown that evolutionary switches between specialism and generalism are common (Desdevises et al. 2002, Poulin et al. 2006, Johnson et al. 2009, 2011), and experimental and observational studies have documented adaptive host range expansions and host switches (e.g. Ebert 1998, Tanaka et al. 2007, Magalhães et al. 2014, Cenzer 2016).

Parasite specialization is expected to be shaped by the heterogeneity of the host population, and by the magnitude of the fitness trade-offs in host use (Kassen 2002). Successfully infecting, exploiting, and evading the immune system of different host species is a challenging endeavor, and often cannot be done with perfect success. When improvements in one host trade-off with improvements in another (e.g. Ebert 1998, Turner and Elena 2000, Agudelo-Romero et al. 2008, Legros and Koella 2010), the strength of the trade-off modulates the evolution of the parasite's niche breadth, which would otherwise evolve to match the degree of host heterogeneity (Kassen 2002, Gandon 2004). Strong trade-offs select for specialists, while weak trade-offs allow the evolution of generalists (Regoes et al. 2000, Gandon 2004, Ravigne et al. 2009).

Despite their ubiquity, the evolution of multi-host parasites remains poorly understood. It is clear that trade-offs in fitness across hosts are not universal (Fry 1996, Bedhomme et al. 2005, e.g. Nidelet and Kaltz 2007, Magalhães et al. 2009, Messina and Durham 2015), although costs of generalism are regularly found (e.g. Poullain et al. 2008, Legros and Koella 2010, Bruns et al. 2014); thorough empirical studies of parasite life history and virulence across multiple hosts are rare (reviewed by Rigaud et al. 2010).

In this section

In Aigues-Mortes, the invasion of *A. franciscana* extended the potential host range of the system's various parasites. In response, the microsporidians *A. rigaudi* and *E. artemiae* have indeed established regular infections of their novel hosts – *A. franciscana* for *A. rigaudi*, and possibly both *Artemia* species for *E. artemiae* (Rode et al. 2013c). We therefore expect their life history and evolutionary potential to reflect this shift in host range. *F. liguloides*, in contrast, has only rarely been reported to infect *A. franciscana* (Georgiev et al. 2007, 2014, Redón et al. 2015a), and never in Aigues-Mortes (Sánchez et al. 2012, Rode et al. 2013b); the factor under selection should therefore be the ability to establish an infection in the novel host (see Appendix 2).

In this section, I investigate the causes and consequences of specialization in the microsporidians *A. rigaudi* and *E. artemiae*.

- **Chapter 3: Cryptic and overt host specificity shapes the epidemiology of two sympatric microsporidian species.** This study uses field and lab data to investigate the prevalence dynamics of *A. rigaudi* and *E. artemiae* in the field. It demonstrates that, although the two microsporidians can and do infect both hosts, each is specialized to some degree: *A. rigaudi* is preferentially associated with *A. parthenogenetica*, and *E. artemiae* with *A. franciscana*. Furthermore, *A. rigaudi* is dependent on the presence of *A. parthenogenetica* to persist in the population.

The seasonal dynamics described in this chapter are also related to the patterns of winter survival described in Appendix 1.

- **Chapter 4: Infectivity, virulence and transmission in a two-host, two-parasite system.** In this chapter, we confirm the partial specialization inferred in Chapter 3, and study its effects on infectivity, virulence, and transmission rate in the two host species. Notably, large-scale experiments suggest that the microsporidians use different mechanisms of host exploitation, causing severe immunopathology and maladaptive virulence in their non-associated hosts.
- **Chapter 5: Specialized host exploitation constrains the evolution of generalism in microsporidian parasites of *Artemia*.** In this study, we investigate the response of *A. rigaudi* and *E. artemiae* to serial passaging on *A. franciscana*, *A. parthenogenetica*, or an alternation of the two. Comparing the infectivity, virulence, and spore production of the evolved lines reveals that infectivity responds well to selection for generalism. However, this effect is outweighed by the inflexible specialization of spore production (a proxy for host exploitation).

Some additional results from this experiment are provided in Appendix 3.

Additional parasite-oriented information is provided in the following appendices:

- **Appendix 1: Survival of microsporidian spores at cold temperatures & spore dormancy.**
- **Appendix 2: Colonization of *Artemia franciscana* by the cestode *Flamingolepis liguloides*.**
- **Appendix 3: Relationships between fitness, infectivity, virulence, and spore production in the serial passage experiment.**

CHAPTER 3

CRYPTIC AND OVERT HOST SPECIFICITY SHAPES THE EPIDEMIOLOGY OF TWO SYMPATRIC MICROSPORIDIAN SPECIES

Eva J.P. Lievens, Nicolas Olivier Rode, Julie Landes, Adeline Segard, Roula Jabbour-Zahab,
Yannis Michalakis, Thomas Lenormand

Identifying the causative factors of an observed disease dynamic is often challenging, especially in seasonal and multi-host systems. In this paper, we use a combination of experiments and long-term field data to disentangle the effects of seasonality and host specificity on prevalence in a simple host-parasite community. We studied the horizontally transmitted microsporidians *Anostracospora rigaudi* and *Enterocytozpora artemiae* in their brine shrimp hosts, *Artemia franciscana* and *Artemia parthenogenetica*, in the salterns of Aigues-Mortes, France. In the field, prevalence varied between 0% and > 90% in all host × parasite combinations. *A. rigaudi* was strongly seasonal, being highly prevalent in summer and absent in winter. Statistical models revealed that this seasonality was driven by the seasonality of the host *A. parthenogenetica*, which acts as a reservoir; *A. rigaudi* is unable to persist in host communities consisting exclusively of *A. franciscana*. *E. artemiae* occurred throughout the year, and was more prevalent in *A. franciscana*; we were unable to determine if the latter is a reservoir host. We confirmed that these dynamics were independent of seasonal temperature effects using experimental tests of infectivity at 15°C and 25°C. Overall, therefore, the different disease dynamics of the two microsporidians were determined by intrinsic aspects of the host × parasite relationships, not by environmental forcing.

In preparation.

INTRODUCTION

Understanding the dynamics of disease is a major challenge in epidemiology. Disease dynamics, the variation in parasite incidence or prevalence over time, can be driven by a variety of environmental and intrinsic factors. Environmental, or external, factors include climate (Altizer et al. 2006), host behavior (e.g. Hosseini et al. 2004, Altizer et al. 2006, Lass and Ebert 2006), and predator behavior (e.g. Duffy et al. 2005); these effects drive many seasonal patterns of incidence and prevalence. Disease dynamics can also be determined by factors intrinsic to the host-parasite system, such as host immunity and resistance (e.g. Hosseini et al. 2004, Grassly et al. 2005, Duffy et al. 2009), or interactions between host and parasite genotypes (e.g. Decaestecker et al. 2007).

Identifying the causative factors underlying a particular pattern of disease is often difficult (Altizer et al. 2006). Seasonal increases in disease prevalence, for example, could be driven by straightforward effects of temperature on transmission (e.g. Ebert 1995, Dunn et al. 2006), or by correlated changes in e.g. food availability, host density, or host immunity. To sift through these confounding possibilities, time-intensive experiments (e.g. Lass and Ebert 2006, Lass et al. 2011), comparative data (e.g. Grassly et al. 2005), informed modelling (e.g. Hosseini et al. 2004, Altizer et al. 2006), or combinations of these approaches (e.g. Duffy et al. 2005, 2009) are necessary.

The interpretation of disease dynamics is further complicated when the parasite under study is transmitted between multiple host species (a common occurrence, Cleaveland et al. 2001, Taylor et al. 2001, Streicker et al. 2013). In such multi-host systems, the occurrence of the parasite in one host species also depends on its prevalence in other host species (Dobson 2004, Gandon 2004). Changes in incidence or prevalence, therefore, can rarely be understood for one host in isolation. This point is particularly well demonstrated by parasites with ‘reservoir’ and ‘spillover’ hosts (Viana et al. 2014). Reservoirs are host species or species complexes in which the parasite can persist indefinitely (i.e. is endemic), and which transmit infection to ‘spillover’ hosts, who provide little or no transmission for the parasite (Ashford 1997). In these cases, the parasite’s prevalence in the spillover hosts is determined by its prevalence in the reservoir (e.g. Dobson 1995, Rhodes et al. 1998, Power and Mitchell 2004, Nugent 2005); ignorance of the latter will lead to misinterpretation of the former.

In this paper, we study the disease dynamics of two parasite species in a seasonal, multi-host system. In the saltern of Aigues-Mortes in Southern France, the brine shrimp *Artemia franciscana* and *Artemia parthenogenetica* and their microsporidian parasites *Anostracospira rigaudi* and *Enterocytozpora artemiae* form a two-host, two-parasite system (Rode et al. 2013a). We used long-term prevalence data and experimental tests of microsporidian infectivity to explore the disease dynamics and host specificity of *A. rigaudi* and *E. artemiae*. We show that while *A. rigaudi* is strongly seasonal, it is host specificity that is the main driver of field prevalence in both microsporidians.

METHODS

Host-parasite system

Artemia (Branchiopoda: Anostraca), also called brine shrimp, is a genus of small crustaceans whose members populate salt lakes and salterns around the world. Along the Mediterranean coast of France, two *Artemia* species coexist: *A. parthenogenetica* and *A. franciscana*. *A. parthenogenetica* is a parthenogenetic clade native to the area, while *A. franciscana* is a bisexual species native to the New World (Thiéry and Robert 1992, Amat et al. 2005). *A. franciscana* has been present in this region since 1970 (Rode et al. 2013c).

A. rigaudi and *E. artemiae* are microsporidian parasites of *Artemia* (Rode et al. 2013a). Although common (Rode et al. 2013c), they have only recently been described and little is known about their ecology. Both species parasitize the gut epithelium and are continuously transmitted to new hosts via free-living spores (Rode et al. 2013b, 2013a). Evidence from wide-ranging sampling suggests that *A. rigaudi* is native to the Old World, and presumably evolved with Old World species such as *A. parthenogenetica*, while *E. artemiae* also occurs in the New World, where the sole available host is *A. franciscana* (Rode et al. 2013c). Accordingly, in a ‘snapshot’ sampling effort of the Mediterranean coast, *A. rigaudi* was found to be more prevalent in *A. parthenogenetica*, while *E. artemiae* was more prevalent in *A. franciscana* (Rode et al. 2013c).

Field data: microsporidian prevalence in a metacommunity

Characteristics of the Aigues-Mortes saltern

We studied *A. rigaudi* and *E. artemiae* infecting *A. franciscana* and *A. parthenogenetica* in the saltern of Aigues-Mortes in Southern France (43.53°N, 4.21°E). This is a seasonal system, where temperature, salinity, and species composition vary throughout the year. Average monthly temperature ranges from 5-10°C in winter (December to February) to 20-25°C in summer (June to September, Fig. 1B). The salinity is highly variable, ranging from roughly 50 to roughly 250 g salt/L, but is generally higher from May to November than from January to April (Fig. 1A; we have no data for December). *Artemia* are present year-round in large quantities (the population size is estimated to be on the order of 10^9 – 10^{10} by commercial exploiter F. Gout, Camargue Pêche, Grau-du-Roi, France; personal communication), but their density is typically low in late winter and early spring (personal observation). Finally, the species composition of the *Artemia* population varies: *A. parthenogenetica* are entirely absent in winter, but form the majority of the population in summer (Fig. 1C).

From a microsporidian perspective, the Aigues-Mortes saltern is a metacommunity. It is made up of a network of large basins, between which water is allowed to flow or not as a function of the salt production process. This causes environmental factors such as salinity and food quality to vary, leading to variation in the outcomes of inter-host competition: *A. franciscana* or *A. parthenogenetica* can outcompete one another or coexist (Browne 1980, Browne and Halaných

1989, Barata et al. 1996b). At any given time, therefore, adjoining basins can contain different host communities, though water movement between the basins is regular enough that there is no spatial structure in the *Artemia* population (Nougu   et al. 2015). The sites Fangouse, Puit Romain, and Site 9 are isolated from the general flow of water (though other types of dispersal are possible for *Artemia*, e.g. Brendonck and Riddoch 1999, Green et al. 2005). Within basins, *Artemia* communities are also well-mixed (Lenz and Browne 1991), so that we can assume that all the spore pools of *A. rigaudi* and *E. artemiae* spores are shared among the host species (Fels 2006).

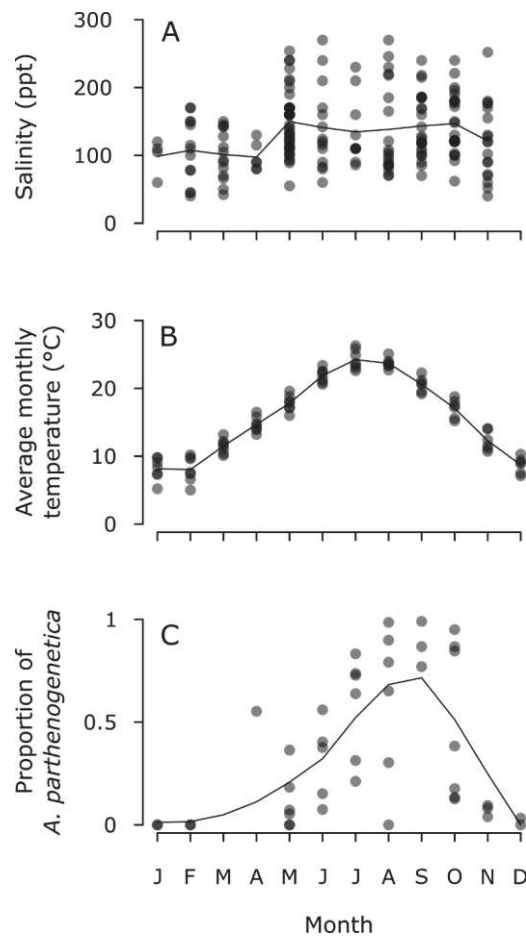


Figure 1. Seasonality in the Aigues-Mortes saltern. A) Average monthly temperature between 2008 and 2015. Temperature data was collected at the nearby meteorological station Le Grau-du-Roi – Repausset-Levant and made publicly available by the organization Infoclimat (Association Infoclimat 2001). The line traces the mean across years. B) Salinities recorded by our team at various points in the Aigues-Mortes saltern between 2008 and 2015. Each point represents one observation; the line traces the mean salinity per month. C) Species composition of the *Artemia* population, expressed as the proportion of the population that is *A. parthenogenetica* (figure reprinted from Lievens et al. 2016). Each point represents one sample; overlapping points shade to black. All but two of the samples were collected in Aigues-Mortes, the remaining two were collected in Gruissan, France (roughly 100 km South-West of Aigues-Mortes). The line represents a 2nd-degree polynomial local regression (LOESS) fitting.

Prevalence data collection

We obtained prevalence data for *A. rigaudi* and *E. artemiae* from 94 samples of *Artemia*, collected at 14 different sites in the Aigues-Mortes saltern between 2008 and 2015. Individuals in the samples were either tested for the presence of *A. rigaudi* and *E. artemiae* immediately after collection, or stored in 96% ethanol and tested later; this prevented any infection-specific mortality from skewing the results. There was no visible swarming behavior at any of the sampled locations at the time of collection (swarming skews prevalence patterns, Rode et al. 2013b).

A random subset of adult *A. parthenogenetica* and/or *A. franciscana* in each sample was tested for the presence of *A. rigaudi* and *E. artemiae*. Not all samples contained both *A. parthenogenetica* and *A. franciscana*; in samples which did, we usually, but not always, tested for infection in both host species. On average, we tested 26.8 individuals per sample (min = 1, max = 182, standard deviation = 24.3). Testing was done by PCR using species-specific microsporidian primers, following Rode et al. (2013a).

Sample-specific environmental and demographic data

In addition to prevalence data, we had environmental and demographic data for many of the samples; we call these variables the sample-specific factors.

First, we knew whether each sample came from a low-, middle- or high-salinity site. The actual salinity at any given site varies seasonally, but can also change dramatically from one day to the next if the water flow in the saltern is redirected. However, the structure of the saltern means that the salinity at some sites is always lower, higher, or roughly equal to the average salinity of the saltern at that time. We call these the low-salinity (Caitive Sud, Pont de Gazette, Site 1, Site 3, Site 8, Site 12, St-Louis), middle-salinity (Caitive Nord, Puit Romain, Site 4) and high-salinity sites (Fangouse, Site 9, Site 10), respectively. This relative salinity classification acts as a residual of salinity after seasonal effects are taken into account, and is not sensitive to the large variability of the actual salinity measures. For 8 samples the sampling site was not known, and we could not assign a classification to these sites.

Second, we had information on the species composition for 93 of our 94 samples. 65 samples contained both *A. franciscana* and *A. parthenogenetica*; 25 samples contained only *A. franciscana*; and three samples contained only *A. parthenogenetica*.

Statistical analyses

For each microsporidian species, we did a two-part statistical analysis. First, we described the general shape of the prevalence curves through time. Second, we tried to explain the variation around the general curves by including sample-specific effects. All analyses were done with generalized linear mixed models in R version 3.1.3 (package “lme4”, Bates et al. 2015, R Core Team 2015), with the number of infected vs. non-infected hosts as the (binomial) response

variable. We included *Sample* as a random effect to control for the non-independence of *A. franciscana* and *A. parthenogenetica* from the same sample. We did not include *Site* in our analyses because working salterns regularly re-distribute the water between basins, so *Artemia* sampled from the same site may or may not be from the same subpopulation. Model comparison was done using the corrected AIC (Hurvich and Tsai 1989).

We first described the general shape of the prevalence curve through time, using only sampling *Month*, *Host species*, and the interaction between the two as possible fixed effects. In this way, we simply describe the temporal dynamics and host associations of each parasite, without trying to identify the underlying causative factors. We compared full models with linear, quadratic, and exponential *Month* terms, then used the best-fitting term to select the fixed effects. The resulting optimal models will be called the ‘general models’ in the following paragraphs.

Second, we tried to explain the variation around the prevalence curves by including sample-specific effects. If adding species composition or salinity information to the general model for the prevalence curve explained more of the variation in the data, this would imply an independent and potentially causative effect of this factor. For each microsporidian species, we thus compared the fit of the general model to that of the general model with added terms for *Presence of other host species* and *Relative salinity*, as well as the possible interactions. Because we had no power to test the effect of *A. franciscana* presence on microsporidian prevalence in *A. parthenogenetica* (the latter was only present by itself in three samples), we only analyzed the data for *A. franciscana*. We further restricted our dataset to sites where the salinity classification and the species composition were known; this left us with 62 samples. The full models were heavily overdispersed, so we included individual-level random effects in the subsequent models.

In addition, we tested whether coinfection rates (with both *A. rigaudi* and *E. artemiae*) were significantly higher or lower than expected. We used Cochran-Mantel-Haenszel tests (package “stats” in R version 3.1.3, R Core Team 2015) to test the independence of *A. rigaudi* and *E. artemiae* prevalence across samples. We performed separate tests for coinfection in *A. franciscana* and *A. parthenogenetica*.

Experimental data: microsporidian infectivity

We did two experiments testing the infectivity of *A. rigaudi* and *E. artemiae* in each host. In both cases, we detected infection by PCR using the protocol described by Rode et al. (2013a), and we relied on their finding that an infection is detectable 5-6 days after the host has been exposed.

Exp. 1: Effect of temperature, host species and host genotype on transmission

We tested the effects of temperature and host species on parasite transmission by exposing non-infected *A. franciscana* or *A. parthenogenetica* to *A. rigaudi* and *E. artemiae* at 15 or at 25°C.

The experimental hosts were adult *Artemia* pulled from laboratory stock collections of Aigues-Mortes lineages. We formed recipient groups based on genetic background. For *A. parthenogenetica*, prior evidence suggests that microsporidian infectivity depends on host genotype (Rode et al. 2013b), so the recipient groups were formed by collecting individuals from two families of each of four isofemale lines, P6 to P9. The recipient groups for *A. parthenogenetica* were named P6.1, P6.2, P7.1, P7.2, P8.1, P8.2, P9.1, and P9.2. For *A. franciscana*, four families, F1 to F4, were combined to form four recipient groups: F1+3, F2+3, F3, and F4.

From each recipient group, 10 individuals were infected at 15°C, 10 individuals were infected at 25°C, 5 individuals served as negative controls at 15°C, and 5 individuals served as negative controls at 25°C. The recipient individuals were infected via exposure to infected ‘donor’ hosts. Donor hosts were a mixed group of *A. parthenogenetica* and *A. franciscana*, collected from sites in the Aigues-Mortes saltern with highly prevalences of *A. rigaudi* and *E. artemiae*. Groups of 15 donors were placed in strainers above the jars of 10 recipient hosts. This allowed spores to pass through, but kept donors and recipients from mixing (Rode et al. 2013b). The strainers were rotated every 45 minutes to ensure randomized exposure. Exposure lasted for 9 hours, followed by a 6-day incubation period. Each jar was fed 20 mL of algal solution at the start of the exposure period, then fed 15 and 30 mL of algae on days 1 and 2 of the incubation period. On day 6 of the incubation period, the surviving individuals were sacrificed and tested for the presence of *A. rigaudi* and *E. artemiae*.

To analyze the effect of host species, host genotype, and temperature on the probability of detecting an infection with *A. rigaudi* or *E. artemiae*, we used generalized linear mixed models in R version 3.1.3 (package “lme4”, Bates et al. 2015, R Core Team 2015), with the infection status of each individual as the (binary) response variable. We included *Jar* as a random effect to control for the non-independence of the individuals sharing the same jar; this effect was nested within *Recipient group* to control for the non-independence of individuals with the same background. The possible fixed effects were *Temperature* and *Species/Genotype* and their interaction. *Species/Genotype* separated the individuals into *A. franciscana*, *A. parthenogenetica* P6, *A. parthenogenetica* P7, *A. parthenogenetica* P8, and *A. parthenogenetica* P9. We separated the *Artemia* this way because we expected to find differences among the clones (Rode et al. 2013b), whereas the *A. franciscana* families had been mixed for the experiment and were thus indistinguishable. The significance of the predictors was tested using the likelihood ratio test.

Exp. 2: Effect of temperature and incubation time on *A. rigaudi* detection

In experiment 1, both microsporidians had low transmission success after 6 days at 15°C (see Results). However, the factors underlying this temperature effect were unclear: the reduced transmission could have been caused by a direct effect of temperature on the microsporidians (e.g. on spore germination, Undeen et al. 1993), or by indirect effects of temperature on the ectothermic *Artemia*. Cool temperatures lower the metabolic rate of *Artemia* (Engel and Angelovic 1968), thereby also lowering their defecation and ingestion rates (which would have reduced the effective inoculum size, Burns 1969, Larsen et al. 2008) and dampening their cellular metabolism (which would have slowed the accumulation of microsporidian DNA in the host, e.g. Dunn et al. 2006). Because *E. artemiae* was present in the field in winter (see Results), we could infer that its low transmission success at 15°C must be due, at least in part, to indirect effects. However, for *A. rigaudi*, which was not found in the field in winter (see Results), it was important to disentangle these confounding effects. To do this, we tested the effect of temperature and incubation time on (detected) infectivity, while controlling the spore dose. We limited this experiment to testing *A. rigaudi* infecting *A. franciscana*.

In this experiment, we allowed *A. rigaudi* infections to incubate at 15°C or 25°C for different lengths of time. We exposed uninfected *A. franciscana* to feces (containing spores) collected from a laboratory stock of *Artemia* infected with *A. rigaudi*; the spore concentration was unknown. Exposure occurred in groups: six groups of 20 hosts were placed in 50 mL autoclaved brine at 90 g salt/L and 2.8 mL feces solution was added. Four groups were exposed at 15°C, while two groups were exposed at 25°C. Exposure lasted two days, during which time all spores could be ingested (Reeve 1963b). After two days, hosts were separated and each individual was placed in a hemolymph tube containing 2.5 mL brine; this prevented second-hand infections later in the experiment. The infection was allowed to incubate at the exposure temperature for four days, after which half of the surviving hosts from each group were sacrificed, and two of the groups exposed at 15°C were moved to 25°C. After a further six days of incubation, all remaining hosts were sacrificed and tested for the presence of *A. rigaudi*. Hosts were fed 0.25 mL *Tetraselmis chuii* solution (6.8×10^9 cells/L, Fitoplancton marino, Spain) every two days. All hosts were adults collected from laboratory stock populations of uninfected *A. franciscana*.

We analyzed the proportion of individuals who tested positive for *A. rigaudi* using a generalized linear model with a binomial response variable (package “lme4”, Bates et al. 2015, R Core Team 2015). Because the sensitivity of our PCR is fixed, an increase in detectability over time reflects an increase in the quantity of parasite DNA present in the host (i.e. intra-host reproduction). We used *Periods incubating at 15°C* and *Periods incubating at 25°C* as fixed effects (with one period equal to six days), with *Host group* as a random variable controlling for pseudo-replication. To test for an effect of incubation temperature, we also compared this model to a model containing *Time incubating* as its only fixed effect. Model comparison and parameter testing were done using the likelihood ratio test.

RESULTS

Field data: microsporidian prevalence in a metacommunity

First, we described the prevalence dynamics for *A. rigaudi* and *E. artemiae* throughout the year (Fig. 2). *A. rigaudi* was strongly seasonal: it was highly prevalent from August to October, but absent in winter (quadratic effect of *Month*, Supp. Table 1). These seasonal dynamics were not different for *A. rigaudi* in the two hosts, but its prevalence was higher in *A. parthenogenetica* (effect of *Host species*, Supp. Table 1). The prevalence of *E. artemiae* was highly variable and this microsporidian was not strongly seasonal, nevertheless there was statistical support for temporal effects (quadratic effect of *Month*, Supp. Table 1). The precise dynamics depended on the host species: *E. artemiae*'s prevalence increased more steeply towards the end of the year in *A. parthenogenetica* than in *A. franciscana* (interaction between *Host species* and *Month* effects, Supp. Table 1). Overall, *E. artemiae* was more prevalent in *A. franciscana*.

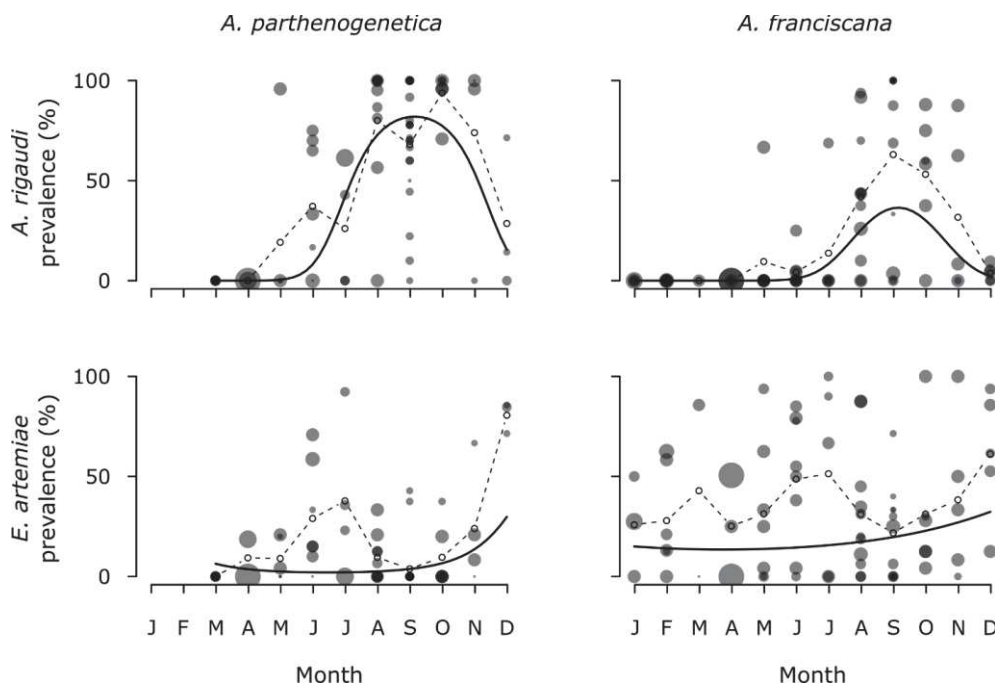


Figure 2. General prevalence patterns of *A. rigaudi* (top) and *E. artemiae* (bottom) in *A. parthenogenetica* (left) and *A. franciscana* (right). Solid dots are samples; the area of the dot represents the number of individuals in the sample. Overlapping dots shade to black. Solid line: predictions of the best general model; dashed line with open circles: mean prevalence across samples. There is no prevalence data for *A. parthenogenetica* in January and February because this species is absent in winter.

Next, we introduced sample-specific effects to the general models, in order to explain some of the variation around the prevalence curves. We tested for effects of salinity and the presence of *A. parthenogenetica* on the prevalence of the microsporidians in *A. franciscana*. For *A. rigaudi*, the species composition had a strong effect (Fig. 3, Supp. Table 2): in the absence of *A. parthenogenetica*, the prevalence of *A. rigaudi* was almost always 0%, and never higher than 10%. In contrast, in the presence of *A. parthenogenetica* the prevalence could be very high, and

the seasonal dynamics found in the general model reappeared. There was mixed support for an additional effect of salinity classification, with the high-salinity sites typically having lower prevalences. For *E. artemiae*, the species composition had no effect, and there was only weak support for an effect of salinity classification (Supp. Table 2).

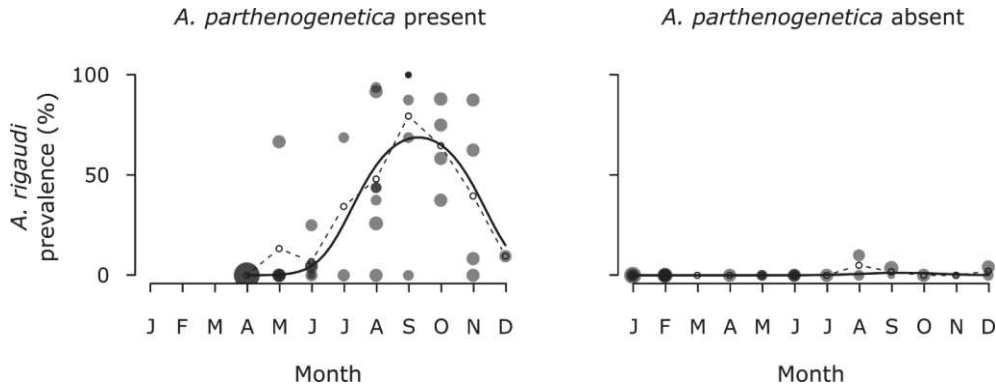


Figure 3. Prevalence of *A. rigaudi* in *A. franciscana* when *A. parthenogenetica* is present (left) or absent (right). Taken together, these two plots are equivalent to the top right plot of Fig. 2. Solid dots are samples; the area of the dot represents the number of individuals in the sample. Overlapping dots shade to black. Solid line: predictions of the best sample-specific model; dashed line with open circles: mean prevalence across samples.

Experimental data: microsporidian infectivity

Exp. 1: Effect of temperature, host species and host genotype on transmission

The P8 genotype of *A. parthenogenetica* and the F4 family of *A. franciscana* were previously infected (all controls tested positive for *A. rigaudi* and *E. artemiae*, respectively), so these recipient groups were removed from the analysis. The previous infections appeared to have an inhibitory effect: none of the exposed P8s were infected with *E. artemiae* at the end of the experiment ($n = 38$), and none of the exposed F4s were infected with *A. rigaudi* ($n = 21$).

Temperature had a clear effect on the probability of infection of both *A. rigaudi* and *E. artemiae*; neither infected well at low temperatures ($p < 0.001$ in both cases, Fig. 4). *A. rigaudi* infected all three genotypes of *A. parthenogenetica* and *A. franciscana* equally well, but infectivity was dependent on *Species/Genotype* in *E. artemiae* ($p < 0.01$). Post-hoc Tukey tests indicated that *A. franciscana* and P9 were equally susceptible to *E. artemiae*, while P6 and P7 were much less susceptible ($p < 0.01$).

Exp. 2: Effect of temperature and incubation time on A. rigaudi detection

The probability of detecting *A. rigaudi* in *A. franciscana* increased with incubation time at both 15°C and 25°C ($p = 0.02$ and $p < 0.001$, respectively, Fig. 5), indicating that there was a significant accumulation of parasite DNA within the host during incubation. Parasite reproduction was faster at 25°C ($p < 0.001$). Therefore, the apparent reduction in infectivity of *A. rigaudi* at 15°C during experiment 1 was at least partly caused by slower parasite reproduction inside the hosts, delaying its detectability. The effect size in this experiment,

however, was not as large as that in experiment 1. This difference could be explained by a higher initial spore dose in experiment 1, which would have a disproportionate effect on prevalence at 25°C if we assume exponential parasite growth. A separate, direct effect of temperature on infectivity is unlikely, because exposure at 15°C did not affect the prevalence of infection in treatments that were incubated at 25°C for one period (Fig. 5, compare rightmost square and triangle).

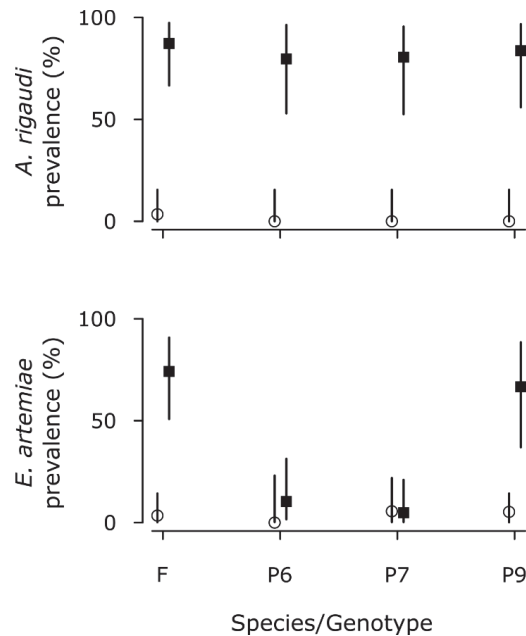


Figure 4. Infectivity of *A. rigaudi* (top) and *E. artemiae* (bottom) as a function of temperature and host type. Individuals were sacrificed and tested after six days of exposure and incubation at 15°C (open circles) or 25°C (filled squares). Species/Genotypes were *A. franciscana* (F) and *A. parthenogenetica* isofemale lines P6, P7 and P9. Vertical lines represent the 95% confidence intervals.

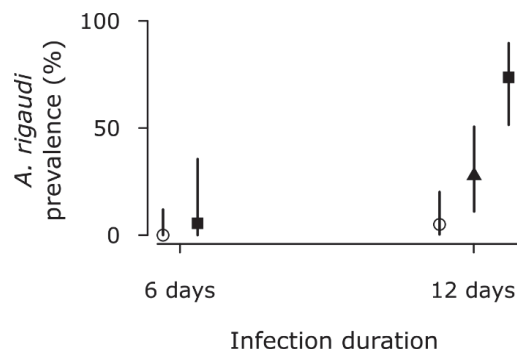


Figure 5. Detection of *A. rigaudi* infections in *A. franciscana* as a function of temperature. Groups of individuals were exposed to *A. rigaudi* and maintained at 15°C (open circles) or 25°C (filled squares) for six or twelve days, after which they were sacrificed and tested. The filled triangles represent a group which was exposed and maintained at 15°C for the first six days, and then moved to 25°C for the remaining six days. Vertical lines represent the 95% confidence intervals.

DISCUSSION

We studied the prevalence of *A. rigaudi* and *E. artemiae*, two ecologically similar microsporidian parasites of the brine shrimp *A. parthenogenetica* and *A. franciscana*, using long-term field data from a meta-community and experimental tests of infectivity. Our data revealed that host specificity is the main driver of prevalence in *A. rigaudi* and *E. artemiae*. Because coinfection rates were mostly independent, we discuss the two parasite dynamics separately.

Difference in seasonal dynamics is not due to temperature-sensitivity

Despite their ecological similarity, *A. rigaudi* and *E. artemiae* have very different yearly dynamics (Fig. 2). *A. rigaudi* is strongly seasonal, being completely absent in winter and highly prevalent in late summer and autumn. In contrast, the prevalence of *E. artemiae* is largely random, with no overarching seasonal pattern. At first glance, therefore, it could appear that *A. rigaudi* is sensitive to low temperatures, while *E. artemiae* is not. However, *A. rigaudi* is able to infect at 15°C (although those infections were not immediately detectable, Fig. 5), so temperature-sensitivity cannot entirely explain their different seasonal patterns. Instead, as discussed in the next section, *A. rigaudi*'s cyclical dynamics are driven by the seasonality of *A. parthenogenetica* (compare Fig. 2 and Fig. 1C), and therefore by host specificity.

A. franciscana is a spillover host for A. rigaudi

Unexpectedly, *A. rigaudi* is not the generalist parasite it appears to be at first glance (Fig. 2). In the lab, the parasite infects both hosts equally well (Fig. 4). However, a more detailed analysis of our field data revealed that *A. rigaudi* cannot persist on *A. franciscana* in the absence of *A. parthenogenetica* (Fig. 3). The presence of *A. rigaudi* in *A. franciscana* must therefore be dependent on regular re-introduction from *A. parthenogenetica*; the former is a spillover and the latter a reservoir host (Ashford 1997).

Spillovers from *A. parthenogenetica* to *A. franciscana* clearly occur frequently enough that *A. rigaudi* appears to be independently present in both hosts (Fig. 2). Some authors have termed such frequent spillovers 'apparent multi-host' or 'pseudo-endemic' dynamics (Fenton and Pedersen 2005, Viana et al. 2014s; for other examples see Dobson 1995, Rhodes et al. 1998). Pseudo-endemic spillover dynamics are particularly hard to identify, because they are difficult to distinguish from true endemism (Viana et al. 2014). This makes the danger of misinterpreting the epidemiology and evolution of pseudo-endemic parasites very high, if their community context is not investigated (Power and Mitchell 2004). For example, previous information led Rode et al. (2013c) to conclude that *A. franciscana* could be a winter reservoir for *A. rigaudi*. In addition, based only on its overall prevalence, we could have parsimoniously interpreted *A. rigaudi*'s seasonal dynamics as a reflection of temperature sensitivity instead of host specificity (see above).

Spillover hosts are, in effect, ecological sinks: they provide little or no transmission for the parasite, and require regular reintroduction from the reservoir host to maintain infection (Holt and Hochberg 2002, Dennehy et al. 2006). A host can be a sink for a parasite if its susceptibility, abundance, and/or lifetime spore production are low (Holt and Hochberg 2002, Dobson 2004, Streicker et al. 2013). For *A. franciscana* and *A. rigaudi*, our experiments ruled out the first possibility (*A. rigaudi* infected both hosts equally well in the lab, Fig. 4), and although our dataset does not contain demographic information, we think a dearth of hosts is unlikely. *Artemia* biomass is mainly constrained by food availability (Browne 1980), temperature (Barata et al. 1996a), and salinity (Wear and Haslett 1986), although the effects of these environmental factors are species-dependent. Nevertheless, we consider that in a given season, host abundance should not be lower in sites without *A. parthenogenetica* than in sites with *A. parthenogenetica*. We therefore speculate that *A. franciscana* is a poor host for *A. rigaudi* because infected hosts produce few spores. Experimental studies will be needed to confirm this.

The source-sink dynamics of *A. rigaudi* reflect the evolutionary history of the Aigues-Mortes system, in which *A. parthenogenetica* and *A. rigaudi* coexisted long before the introduction of *A. franciscana* (in 1970, Rode et al. 2013c). Initially therefore, *A. rigaudi* would have been well-adapted to its historical host, infecting the novel host only incidentally (Agosta et al. 2010). It is interesting that after ± 45 years of evolution in a two-host system, in which the novel host is often the most abundant (Fig. 1C), *A. rigaudi* has not become a true generalist. This may be explained by the difficulties involved in out-of-niche evolution (Lenormand 2002). Specifically, in parasite systems with reciprocal transmission between the reservoir and the spillover host (also called spillback, Nugent 2011), adaptation to the spillover host is constrained by the costs of generalism (Benmayor et al. 2009). Generalism can be costly if the novel host is of lower quality, or if a higher fitness in the novel host is associated with a reduced fitness in the original host (Leggett et al. 2013). This can lead to very stringent conditions for the evolution of generalist parasites (Benmayor et al. 2009). Furthermore, adaptation is less likely when the spillover host is less abundant (Antonovics et al. 2002, Holt and Hochberg 2002). Together, these factors may explain why *A. rigaudi* does not switch hosts: in summer, *A. parthenogenetica* are abundant and the cost of generalism may be high; in winter, there is no cost of generalism because only *A. franciscana* is present, but the host population is orders of magnitude smaller than it is in summer (Rullman et al., unpublished data). Winter temperatures could also prevent adaptation by slowing parasite growth, thus reducing the number of parasite generations (Fig. 5) (Dunn et al. 2006).

Finally, depending on the depth of the sink, the presence of the spillover host *A. franciscana* could provide a hidden advantage for *A. parthenogenetica*. Hosts that absorb more free-living parasite stages than they produce over their lifetime can act as 'inhibitory' hosts (Holt et al. 2003), reducing the force of infection acting on the reservoir (the 'dilution effect', Norman et al. 1999, Ostfeld and Keesing 2000). In *Daphnia*, which like *Artemia* are non-selective filter-

feeders (Reeve 1963a), the presence of less competent hosts has indeed been shown to reduce the prevalence of a fungal parasite in a key host species (Hall et al. 2009). If *A. franciscana* fulfilled a similar function, we would expect to see a higher prevalence of *A. rigaudi* in *A. parthenogenetica* when *A. franciscana* is absent; unfortunately we did not have enough such samples to confirm this. However, if true, the presence of *A. franciscana* may benefit co-occurring *A. parthenogenetica* by lightening the burden of *A. rigaudi*, paradoxically slowing its own invasion (which is otherwise facilitated by parasite effects, Rode et al. 2013c).

Is A. parthenogenetica a spillover host for E. artemiae?

Our results indicate that *E. artemiae*, while able to infect *A. parthenogenetica*, is reasonably specific to *A. franciscana*. At high temperatures, *E. artemiae* is very infective in *A. franciscana* and the *A. parthenogenetica* genotype P9, but not at all in the genotypes P6 and P7 (Fig. 4). These findings are consistent with the genotype-dependent prevalence data collected earlier by Rode et al. (2013b) in natural populations. Because *A. parthenogenetica* populations are a mix of different genotypes (Nougué et al. 2015), we can expect *E. artemiae* to infect *A. parthenogenetica* less well on average, which is consistent with our field results (Fig. 2).

Based on infectivity, *E. artemiae* is more specific than *A. rigaudi*, which is dependent on one host. This begs the question whether *E. artemiae* is also dependent on a reservoir (in this case *A. franciscana*). Using our dataset, we were unable to analyze the ability of *E. artemiae* to persist on *A. parthenogenetica* because only three of our samples did not contain *A. franciscana*. Although these three samples were *E. artemiae*-free, this may have been due to random chance. In the absence of *A. parthenogenetica*-only samples, methods such as those described recently by Fenton et al. (2015) could clarify *E. artemiae*'s status. These methods quantify each host's contribution to parasite fitness using host abundance, parasite prevalence, and parasite shedding data. In future, we will gather host abundance and spore production data for *E. artemiae*; until then, *E. artemiae* serves as an excellent example of the difficulty of distinguishing true from pseudo-endemism based on observational data (Viana et al. 2014).

CONCLUSION AND PERSPECTIVES

In conclusion, we found that the prevalence patterns of *A. rigaudi* and *E. artemiae* are determined by intrinsic factors, not environmental forcing: *A. rigaudi* is dependent on *A. parthenogenetica*, which is seasonal, so *A. rigaudi* is seasonal as well; *E. artemiae* is more infective to *A. franciscana*, which is present year-round, so *E. artemiae* is not seasonal.

Our study shows the utility of a combined observational and experimental approach to disentangle parasite dynamics in a host metacommunity (see also Hall et al. 2009). By including local patch characteristics – in this case, the composition of the host community – in our global prevalence models, we were able to demonstrate that *A. rigaudi* is dependent on the presence of *A. parthenogenetica* to persist in the host community. Conclusive demonstration

that a host is a reservoir typically requires intensive intervention experiments (e.g. Dobson 1995, Caley et al. 1999, MacInnes et al. 2001, Power and Mitchell 2004, Nugent 2005, Serrano et al. 2011); taking advantage of the natural ‘removal’ of hosts allowed us to confirm the reservoir status of *A. parthenogenetica* much more easily. Finally, using simple experiments, we confirmed that the seasonal dynamics of *A. rigaudi* are driven by the seasonality of its reservoir.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Results of the general models for *A. rigaudi* and *E. artemiae* infecting both hosts. A quadratic effect of *Month* was the best fit for both microsporidians; the full models used afterwards thus included *Month*, *Month*², *Species* and the double interactions. All models also included *Sample* as an individual-level random effect, to control for pseudoreplication. Models were compared using the difference in corrected AIC (ΔAICc); also provided are the Akaike weights (*w*).

Model	Fixed effects	AICc	ΔAICc	<i>w</i>
<i>A. rigaudi</i> : full models with varying forms of <i>Month</i> term				
quadratic	Month + Month ² + Host species + Month : Host species + Month ² : Host species	482.9	0	1.00
linear	Month + Host species + Month : Host species	507.8	24.9	0.00
exponential	exp(Month) + Host species + exp(Month) : Host species	513.9	31.0	0.00
<i>A. rigaudi</i> : best fixed effects structure with quadratic <i>Month</i> term				
1	Month + Month ² + Host species	482.4	0	0.40
2 (full model)	Month + Month ² + Host species + Month : Host species + Month ² : Host species	482.9	0.5	0.31
3	Month + Month ² + Host species + Month ² : Host species	484.4	2.0	0.15
4	Month + Month ² + Host species + Month : Host species	484.5	2.1	0.14
5	Host species	545.8	63.4	0.00
6	Month + Month ²	638.5	156.1	0.00
7	-	707.4	225.0	0.00
<i>E. artemiae</i> : full models with varying forms of <i>Month</i> term				
quadratic	Month + Month ² + Host species + Month : Host species + Month ² : Host species	590.7	0	0.87
exponential	exp(Month) + Host species + exp(Month) : Host species	595.2	4.5	0.09
linear	Month + Host species + Month : Host species	597.1	6.4	0.03
<i>E. artemiae</i> : best fixed effects structure with quadratic <i>Month</i> term				
1 (full model)	Month + Month ² + Host species + Month : Host species + Month ² : Host species	590.7	0	0.90
2	Month + Month ² + Host species + Month ² : Host species	596.2	5.5	0.06
3	Month + Month ² + Host species + Month : Host species	598.5	7.8	0.02
4	Host species	599.2	8.5	0.01
5	Month + Month ² + Host species + Month ² : Host species	601.3	10.6	0.00
6	-	722.9	132.2	0.00
7	Month + Month ²	724.2	133.5	0.00

Supplementary Table 2. Results of the sample-specific models for *A. rigaudi* and *E. artemiae* infecting *A. franciscana*. The full models included the fixed factors of the general model (*Month* + *Month*²; *Species* could not be included because we only used *A. franciscana*), *Presence of A. parthenogenetica*, *Relative salinity*, and their interactions with *Month* and *Month*². All models also included *Sample* as an individual-level random effect, to control for overdispersion. Models were compared using the difference in corrected AIC (ΔAICc); also provided are the Akaike weights (*w*). Only the models that fell within a cut-off value of $\Delta\text{AICc} = 4$ are shown.

Model	Fixed effects	AICc	ΔAICc	<i>w</i>
<i>A. rigaudi</i>				
1	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity	197.3	0	0.23
2	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ²	197.9	0.6	0.17
3	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity + Presence of <i>A. p.</i> : <i>Month</i> ²	199.4	2.1	0.08
4	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity + Presence of <i>A. p.</i> : <i>Month</i>	199.4	2.1	0.08
5	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Presence of <i>A. p.</i> : <i>Month</i> ²	199.9	2.6	0.06
6	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Presence of <i>A. p.</i> : <i>Month</i>	199.9	2.6	0.06
7	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity + <i>Month</i> : Relative salinity	200.0	2.7	0.06
8	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity + <i>Month</i> ² : Relative salinity	200.9	3.6	0.04
9	Presence of <i>A. p.</i> + <i>Month</i> + <i>Month</i> ² + Relative salinity + (<i>Month</i> + <i>Month</i> ²) : Relative salinity	200.9	3.6	0.04
...
General model	<i>Month</i> + <i>Month</i> ²	215.5	18.2	0.00
<i>E. artemiae</i>				
General model	<i>Month</i> + <i>Month</i> ²	347.0	0	0.47
2	<i>Month</i> + <i>Month</i> ² + Presence of <i>A. p.</i>	348.4	1.4	0.23
3	<i>Month</i> + <i>Month</i> ² + Presence of <i>A. p.</i> + Presence of <i>A. p.</i> : <i>Month</i>	350.4	3.4	0.09
4	<i>Month</i> + <i>Month</i> ² + Presence of <i>A. p.</i> + Presence of <i>A. p.</i> : <i>Month</i> ²	350.4	3.4	0.08

CHAPTER 4

INFECTIVITY, VIRULENCE, AND TRANSMISSION IN A TWO-HOST, TWO-PARASITE SYSTEM

Eva J. P. Lievens, Julie M. A. Perreau, Yannis Michalakis[†] and Thomas Lenormand[†]

[†]equal contribution

Although it is widely recognized that many, if not most, parasites infect multiple host species, empirical studies of the effects of multiple host use on parasite life history and virulence remain rare. In this study, we quantified the infectivity, within-host growth, transmission rate, and virulence of two parasites, the horizontally transmitted microsporidians *Anostracospora rigaudi* and *Enterocytozpora 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 that this specialization is associated with suboptimal exploitation, maladaptive virulence, and large costs of resistance in the other host. Our study represents a rare empirical contribution to the study of parasite evolution in multi-host systems, highlighting in particular the difficulties faced by parasites adapting to multiple host species.

In preparation.

INTRODUCTION

It is a recognized challenge for evolutionary parasitology that while most parasites infect multiple host species (Cleaveland et al. 2001, Taylor et al. 2001, Streicker et al. 2013), we are only beginning to explore the ecology and evolution of such systems (Rigaud et al. 2010, Lively et al. 2014). Multi-host parasites evolve in a shifting web of selection pressures, facing the challenge of adapting to hosts that may differ in life history, physiology, immunity, behavior and morphology. This doesn't happen seamlessly. Improvements in one host are often associated with reduced performance in another (e.g. Ebert 1998, Turner and Elena 2000, Kassen 2002, Agudelo-Romero et al. 2008, Legros and Koella 2010), so that we might expect generalism to lead to suboptimal exploitation strategies and virulence in some – or all – hosts (e.g. Poullain et al. 2008, Bruns et al. 2014). The effects of multiple host use on parasite life history and virulence have been considered theoretically (e.g. Regoes et al. 2000, Woolhouse et al. 2001, Gandon 2004), but empirical evidence for these patterns is still very limited (reviewed by Rigaud et al. 2010).

Here, we present a study of parasite life history in a natural multi-host system. In the salterns 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). *A. franciscana* is native to North America, and was introduced to France in 1970 (Rode et al. 2013c). Both *Artemia* species are parasitized by the microsporidians *Anostracospora rigaudi* and *Enterocytozpora artemiae*. These parasites infect the gut epithelium, transmitting infection horizontally through spores released with the feces (Rode et al. 2013b, 2013a). Both microsporidians can attain prevalences of up to 100% in the host community. Evolutionarily, the association of *A. parthenogenetica* and *A. rigaudi* predates the invasion of *A. franciscana*, while *A. franciscana* is also infected by *E. artemiae* in its native range (Rode et al. 2013c). 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.

Given their transmission route and the lack of spatial structure in the saltern (cf. Fels 2006, Noug   et al. 2015), *A. franciscana* and *A. parthenogenetica* become infected through a shared spore pool. 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 relatively specialized (Lievens et al. in prep.a). Lievens et al. showed that *A. rigaudi* is always more prevalent in *A. parthenogenetica*, and is also dependent on this species to maintain itself in the host community. *E. artemiae*, in contrast, is consistently more infectious to and more prevalent in *A. franciscana*.

We studied the infectivity, virulence, and transmission of *A. rigaudi* and *E. artemiae* in each of their hosts. We demonstrate 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*). These findings strongly support the view that adapting to multiple host species is a significant challenge, even when the hosts are common and ecologically similar.

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 condition at 4 °C. We used three batches of cysts, sampled at the sites Cative Nord or Cative 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 maintain the infection. We selected both infected *A. franciscana* and infected *A. parthenogenetica* from the field, and maintained each stock population on a mix of *A. franciscana* and *A. parthenogenetica* hosts (n_{hosts} at any given time = ~20-~50 per microsporidian species). Thus, our stocks contained a mix of spores from different field sites and times, collected from and propagated on both host species. Note that while we tried to

maximize microsporidian diversity through this wide sampling, the genetic diversity of the stocks is unknown.

Spore collection and quantification

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

Experiment 1: Infectivity

Experimental design and execution

Previous papers studied the infectivity of *A. rigaudi* and *E. artemiae* using single, uncontrolled spore doses (Lievens et al. in prep.a, Rode et al. 2013a). Here, we quantified infectivity more precisely by exposing individual *A. parthenogenetica* and *A. franciscana* to a range of controlled spore doses and measuring the proportion of infected individuals. The experiments were not run simultaneously on *A. parthenogenetica* and *A. franciscana*.

We exposed experimental hosts to doses of 0, 400, 800, 1600, 3200 and 6400 spores per individual. To ensure hosts ingested all spores, each host was first exposed in a highly-concentrated medium: individuals were placed in 2 mL Eppendorf tubes with 0.45 mL spore solution, 1 mL extra brine and 0.25 mL algal solution (3.4×10^9 *T. chuii* cells/L deionized water). After two days, hosts were transferred to 40 mL glasses containing 20 mL brine and the infection was allowed to incubate for three more days; hosts were fed a total of 1 mL algal

solution over the three days. Surviving hosts were then sacrificed and tested for the presence of *A. rigaudi* or *E. artemiae* by PCR (following Rode et al. 2013a). Treatments were replicated 20 times, except when spore availability was limiting (*E. rigaudi* on *A. parthenogenetica*: 16, 8 and 4 replicates for the doses 400, 3200 and 6400 spores per individual, respectively). All hosts were ~4 weeks old and measured between 5 and 8 mm; *A. franciscana* hosts were mixed males and females.

Statistical analyses

To analyze the dose-response curves, we used four-parameter log-logistic modeling in R (package “drc”, Ritz and Streibig 2005, R Core Team 2015). In these models, the four parameters determining the shape of the sigmoidal curve are: the lower limit (set to 0 in our case), the upper limit, the slope around the point of inflection, and the point of inflection (which here is the same as the ED₅₀). The (binomial) response variable was the number of individuals that were infected vs. uninfected. Because we did not perform the *A. parthenogenetica* and *A. franciscana* experiments at the same time, we could not control for environmental effects. Thus, we simply tested if the dose-response curves for *A. rigaudi* and *E. artemiae* were different within each host species. To do this, we fit models that did or did not include a ‘microsporidian species’ effect and compared the two using a likelihood ratio test. If the effect was significant, we went on to compare the parameters of the two resulting curves (‘compParm’ function in the drc package).

Experiment 2: Virulence and transmission rate

Experimental design and execution

To quantify the virulence and transmission rates of *A. rigaudi* and *E. artemiae*, we experimentally infected individual *Artemia* with controlled spore doses. We then tracked their survival, growth, reproductive output, and spore production over a two-month period. We also quantified host-to-host transmission at two time points. The experiments were not run simultaneously on *A. parthenogenetica* and *A. franciscana*.

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

Table 1. Number of replicates for the different treatments and blocks. See ‘Methods – Experiment 2’ for more information.

Treatment: [spore dose]	Exposure to <i>A. rigaudi</i>		Exposure to <i>E. artemiae</i>		Controls	
	[3 000 sp/i]		[2 500 sp/i]		[10 000 sp/i]	
<i>A. franciscana</i>	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 ♀
<i>A. parthenogenetica</i>		96 ♀		96 ♀	33 ♀	96 ♀
Batch: 34 ± 2 days old		48 ♀		48 ♀	18 ♀	48 ♀
Batch: 26 ± 2 days old		48 ♀		48 ♀	15 ♀	48 ♀

We exposed experimental hosts to spore doses designed to be comparable while maximizing infection rate (see results of Experiment 1): 3 000 spores/individual for *A. rigaudi* and 2 500 spores/individual for *E. artemiae*. Because *A. parthenogenetica* had low infection rates with *E. artemiae*, a separate set of *A. parthenogenetica* was infected with 10 000 *E. artemiae* spores per individual (Table 1). To ensure hosts ingested all spores, each host was exposed in a highly-concentrated medium over a two-day period: individuals were placed in 2 mL Eppendorf tubes with 0.37 mL spore solution and 1.25 mL brine containing 2.6×10^6 *T. chuii* cells.

After exposure, on day 1 of the experiment, individuals were transferred to open tubes, which rested upright in 40 mL plastic glasses containing 20 mL of brine. The lower end of the tube was fitted with a 1x1 mm net. The netting prevented experimental (adult) individuals from swimming to the bottom of the glass, while allowing spores, feces, and offspring to pass through; this limited secondary infections from a host’s own feces. Glasses were randomly placed in trays (each tray containing 48 glasses), which were routinely rotated to avoid effects of room placement. Water was changed every five days. Hosts were fed 0.5 mL algal solution daily (2.6×10^9 *T. chuii* cells/L deionized water); this feeding regime corresponds to half of the maximum ingestion rate of an adult *Artemia* (Reeve 1963b) and has been shown to reveal energetic trade-offs (Rode et al. 2011). We ended our experiment after 60 days, at which point surviving individuals were sacrificed and tested for infection by PCR (following Rode et al. 2013a).

To quantify the effects of infection on the hosts, we tracked the growth, survival, and reproduction of the experimental individuals. Body length was recorded on days 30 and 60. Survival was recorded daily; dead individuals were tested for infection by PCR (following Rode et al. 2013a). We did not track reproduction for males, because male reproductive success is heavily influenced by the female partner (e.g. female clutch size). For females, measures of reproductive success were recorded daily, including date of sexual maturity (first detection of a fully-formed ovisac or of yolk accumulation in oocytes, Metalli and Ballardin 1970), clutch date, clutch type, and clutch size. *Artemia* females are iteroparous, producing on average one clutch per five days (Bowen 1962, Metalli and Ballardin 1970). Clutches may be of two types: live larvae (‘nauplii’), or dormant encysted embryos (‘cysts’). Neonatal nauplii are barely visible to

the eye and have high death rates in the first few days. For ease of measurement, therefore, clutches of nauplii were counted five days after sighting. During these five days nauplii were in competition for resources with their mother (plus an additional male if *A. franciscana*, see below). However, mothers were removed at each water change, which could happen before the clutch had reached the five-day mark. In these cases, we placed a new tube containing one (or two, if *A. franciscana*) adult male *Artemia* above the nauplii to ensure the same level of food competition.

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

To estimate parasite fitness, we estimated spore production at regular points throughout the experiment. To do this, we collected 1 mL of feces (containing parasite spores) from every experimentally infected host at every water change. Samples were stored in 1.2 mL Qiagen Collection Microtubes and refrigerated until the spore concentration could be quantified. To measure the spore concentration, we homogenized and stained each sample as described above, with minor differences in the centrifugation steps (16 min at 5 000 g) and the final concentration (concentrated to 14.3X by removing 930 μ L of the supernate). Spores were counted once per sample, as described above. Because counting spores is labor-intensive, we restricted our efforts to the feces samples collected on days 15, 30, 45 and 60.

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

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

Statistical analyses: species-level effects

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

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

To estimate the infectiousness of a single spore, we used the results of the transmission assay. To do so, we assumed that the establishment of microsporidian infections follows an independent-action model with birth-death processes. This model assumes that a parasite population grows in the host until it reaches an infective threshold, at which point the infection is considered to be established (Schmid-Hempel 2011 pp. 225–6). In our assay, we considered that an infection was established when we could detect it, i.e. the infectivity threshold corresponds to the threshold for PCR detection (estimated at ~1 000 spores inside the host's body, unpublished data). By explicitly accounting for within-host growth, the birth-death approach circumvents the problem of a short incubation time leading to false negatives for slow-growing infections. In these models, the probability per spore to start an infection, p , is equal to $-\ln\left(\frac{\text{noninfected}}{\text{exposed}}\right)/D$ where D is the spore dose (Schmid-Hempel 2011 pp. 225–6). In our transmission assay, D can be approximated by the number of spores in the fecal sample taken from the donor at the start of the assay (= spore count transformed to spores/mL, or * 700), divided by $5 \times 8 = 40$ (fecal samples accumulated over a 5-day period but we only exposed

Table 2. Overview of statistical analyses; see Supplementary Material for details.

Tested effect	Type of model	Fixed effects	Random/Frailty terms
Virulence of infections: <i>A. franciscana</i> and <i>A. parthenogenetica</i> analyzed separately			
Survival	Parametric survival models ¹ + LRT	Treatment ² , Sex (<i>A. f.</i>), Size class, double interactions	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Growth			
Timing of growth	Paired t-tests	Size on days 1 vs. 30 & 30 vs. 60	
Growth between days 1 & 30	LMM + LRT	Treatment ² * Sex (<i>A. f.</i>) * Size class	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Reproduction ³			
Total number of offspring	Kruskal-Wallis + Dunn ph	Treatment ²	
Time until sexual maturity	Parametric survival models ¹ + LRT	Treatment ² * Size class	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Probability of producing a clutch	Bernoulli GLMM + LRT	Treatment ² * Size class	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Type of offspring produced	Binomial GLMM + LRT	Treatment ² * Size class	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Rate of offspring production ^{4,a}	LMM + LRT	Treatment ² * Size class	Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Timing of offspring production ^{5,a}	Binomial GLMM + LRT	Treatment ² * (Elapsed % of reproductive period + Elapsed % of reproductive period ²)	Individual, Origin (<i>A. f.</i>), Batch (<i>A. p.</i>)
Parasite transmission and fitness: infections of <i>A. franciscana</i> and <i>A. parthenogenetica</i> analyzed together			
Spore production rate ^b			
Spore count ⁶ ~ time ^c	Neg. binomial GLMM + LRT	Day (factorial) * Host sp. * Parasite sp.	Individual
Spore count ⁶ ~ dose ^d	Neg. binomial GLMM + LRT	Day (factorial) * Dose	Individual
Transmission success ~ spore count	Binomial GLMM + LRT	Recipient sp. * Parasite sp. * Spore count	Individual
Infectiousness of one spore, <i>p</i>	LMM + LRT	Recipient sp. * Parasite sp.	Individual
Fitness			
Cumulative spore count	Kruskal-Wallis + Dunn ph	Host-parasite combination ²	
Asymptotic growth rate	Kruskal-Wallis + Dunn ph	Host-parasite combination ²	

Notes: ¹ Best survival distribution chosen by AICc first. 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 maturity. ⁵ Modeled per clutch, as the proportion of the total offspring produced by the host in that clutch, as a function of the elapsed proportion of the reproductive period. ⁶ Spore count = the number of spores counted in the fecal sample; we did not transform the spore count to spores/mL (\approx 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. Kruskal-Wallis + Dunn ph, Kruskal-Wallis tests + Dunn's post-hoc testing. *A. f.*, *A. franciscana*. *A. p.*, *A. parthenogenetica*. sp., species. *, interactions between the factors were included.

recipients for one day; the inoculum was shared amongst 8 recipients). We calculated a value of p for every replicate in the transmission assay.

We used two measures of spore production as overall indicators of parasite fitness. First, we summed the spore counts obtained on days 15, 30, 45 and 60 for each infection; this produced a cumulative spore count. Second, we calculated an asymptotic growth rate for each infection 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 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 cumulative spore count is a measure of the basic reproduction number R_0 , which quantifies 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 within-host population dynamics

The life cycle of *A. rigaudi* and *E. artemiae* is straightforward: after infecting an epithelial cell in the gut of the *Artemia* host, the microsporidian undergoes a series of divisions, terminating in the formation of spores which are released into the gut lumen (Rode et al. 2013a). Both parasites most likely disseminate within the host when spores, after release into the gut, infect new epithelial cells (cf. *Ordospora colligata* & *Glugoides intestinalis*, Refardt and Ebert 2006). (Some microsporidian species use separate spore types for within-host transmission (e.g. Becnel et al. 1989, Agnew et al. 2003), but no additional spore types have been observed for *A. rigaudi* and *E. artemiae* (Rode et al. 2013a).) With this in mind, we can use an increase in the observed spore counts in the feces of individual hosts as a proxy for within-host population growth. We took advantage of this relationship to investigate the within-host population dynamics of *A. rigaudi* and *E. artemiae* in each of their hosts.

In general, the rate of spore production was not monotonic, but increased exponentially, peaked, and decreased (see Results & Supp. Fig. 1). To deal with this time-dependence, we represented each host's spore production curve using three variables: the highest observed spore count (hereafter the 'peak spore count'), the time at which the peak spore count was observed (hereafter the 'peak time'), and the exponential growth rate pre-peak (the 'within-host reproductive rate'). The latter was estimated as the slope of an exponential growth model, fitted through all the coordinates (spore count, i) for which the collection day $i \leq$ peak time. The intercept of the exponential growth model was set to 0 (no spore production at the time of

exposure). We did not examine the post-peak decline in spore count, because it cannot be interpreted as easily with regards to parasite population growth (declines can be caused by a reduced parasite burden or a lower rate of spore maturation, Ebert et al. 2000, Sinden et al. 2007).

First, we examined the factors contributing to within-host reproductive rate. For each host-parasite combination, we constructed linear mixed models containing the fixed effects *Size class*, *Sex* (for *A. franciscana*), and *Spore dose* (for *A. parthenogenetica* infected with *E. artemiae*), all possible interactions, and the random effects *Origin* or *Batch*. All fixed effects combinations were compared using the corrected AIC (Hurvich and Tsai 1989).

Second, we tested for correlations between within-host reproductive rate, peak time, and peak spore count. Correlations were tested using Spearman's ρ and corrected for multiple testing within host-parasite combinations. We used only those individuals for whom we recorded a clear peak, i.e. a maximal spore count followed by a decrease in spore production. This excluded hosts whose peak spore count was simply the last spore count before their death.

Finally, we looked at the relationship between within-host reproductive rate and virulence. To do this, we repeated the survival and reproduction analyses described above for the host-parasite combinations *A. franciscana*-*E. artemiae* and *A. parthenogenetica*-*A. rigaudi* (see Discussion for the rationale behind this choice), with an added *Within-host reproductive rate* term. We added or excluded *Within-host reproductive rate* and its interactions with the other fixed effects, then compared all models using the corrected AIC. This allowed us to see whether the variation in within-host reproductive rate was related to the variation in virulence after the experimentally manipulated factors were taken into account.

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 tested 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* term. We added or excluded this term 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 *Resistant-Infected-Control* 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.

RESULTS

Experiment 1: Infectivity

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

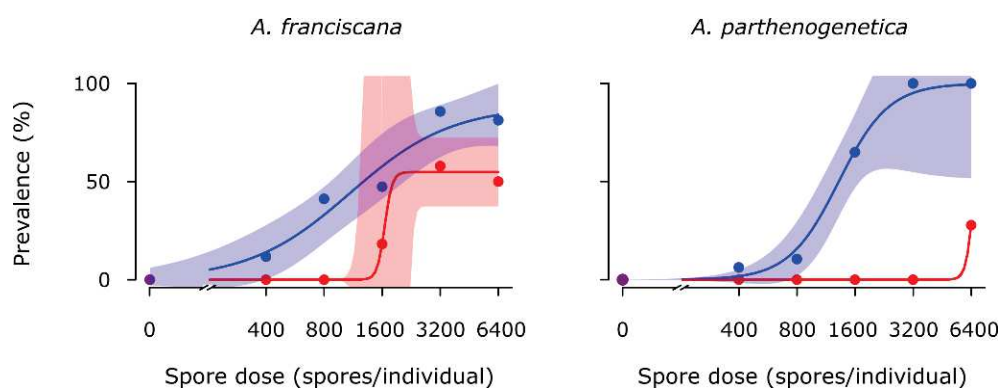


Figure 1. Infectivity of *A. rigaudi* (blue) and *E. artemiae* (red) in *A. franciscana* (left) and *A. parthenogenetica* (right). Points indicate the prevalence (% infected) at each dose; lines are the best fits and the shaded areas represent the 95% CIs. Because the inflection point of *E. artemiae*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

Experiment 2: Virulence and transmission rate

Among host individuals that survived until we could be certain of their infection status (i.e. that survived until at least day 15), infection rates were high (Table. 3). As expected, the detected infection rates were much lower among individuals that died before this threshold day.

Table 3. Detection of infection in individuals that survived until we could be certain of their infection status (i.e. that died after 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	
<i>A. franciscana</i>			
Exposure to <i>A. rigaudi</i>		86 %	50 %
Exposure to <i>E. artemiae</i>		96 %	13 %
<i>A. parthenogenetica</i>			
Exposure to <i>A. rigaudi</i>		100 %	15 %
Exposure to <i>E. artemiae</i>	– low spore dose	64 %	0 %
	– high spore dose	86 %	20 %

Species-level effects

We analyzed the species-level results of Experiment 2 in two parts: first, we examined the virulence of virulence of infections; second, we analyzed parasite transmission and estimated overall parasite fitness. The parasite fitness. The results are summarized in Table 4, and Tables

A synopsis of the virulence results is provided in Table Error! Not a valid bookmark self-reference..

Table 5 and 6 provide brief synopses of the two parts.

First, we analyzed the virulence of parasite infections, expressed as survival, growth, and reproduction (summarized in Table 4). These analyses were run separately for *A. franciscana* and *A. parthenogenetica*. Here, we report only the analyses for infected vs. control individuals, which excluded all individuals that died before day 15. When we compared exposed vs. control individuals that died before the cut-off day the results were not qualitatively different.

In most host-parasite combinations, survival was reduced (Fig. 2). For *A. franciscana*, survival had a lognormal distribution ($\Delta\text{AICc} \geq 4.2$). Infection with a parasite significantly reduced survival ($\chi^2(2) = 49.5, p < 0.0001$); post-hoc testing revealed that this effect was highly significant for *A. rigaudi* and marginally significant for *E. artemiae* ($t = -6.8$ and $-2.2, p < 0.0001$ and $p = 0.05$, respectively). Independently, survival was significantly higher for males ($\chi^2(1) = 34.0, p < 0.0001$) and for individuals from the larger size class ($\chi^2(1) = 4.6, p = 0.03$). For *A. parthenogenetica*, survival was log-logistically distributed ($\Delta\text{AICc} \geq 0.9$). Survival was affected by infection with a parasite, size class, 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

($\chi^2(3) = 19.7, p < 0.001$); 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* (at

Table 4. Summary of the species-level results of Experiment 2. 95% CIs given in brackets. No CIs are reported for analyses based on Kruskal-Wallis tests.

	<i>A. rigaudi</i>	<i>E. artemiae</i>		
<i>A. franciscana</i>				
Virulence of infections (effects compared to controls) ¹				
Survival time (acceleration factor)	0.29 [0.20, 0.41]	0.69 [0.50, 0.96]		
Growth (size difference)	no effect	no effect		
Total number of offspring (median)				
- 2 x nauplii + 1 x cysts	-100%	-62%		
- 1 x nauplii + 1 x cysts	-100%	-63%		
- 1 x nauplii + 2 x cysts	-100%	-73%		
Time until maturity (acceleration factor)	1.53 [1.28, 1.81]	1.18 [1.04, 1.34]		
Probability of reproduction (odds ratio)	0.14 [0.06, 0.28]	no effect		
Clutch type (odds ratio of nauplii vs. cysts)	4.08 [2.13, 8.37]	no effect		
Rate of offspring production (offspring/day)				
- 2 x nauplii + 1 x cysts	no effect	-28% [-45%, -10%]		
- 1 x nauplii + 1 x cysts	no effect	-30% [-47%, -12%]		
- 1 x nauplii + 2 x cysts	-43% [-69%, -8%]	-33% [-51%, -12%]		
Timing of offspring production ²	no effect	no effect		
Parasite transmission and fitness				
Spore production rate (spores/mL/5 days x 700)				
- day 15	0.2 [0.1, 0.3]	4.7 [3.8, 5.9]		
- day 30	3.5 [2.5, 5.1]	45.3 [36.4, 56.4]		
- day 45	13.5 [9.3, 19.7]	33.8 [26.8, 42.7]		
- day 60	21.0 [14.1, 31.4]	13.6 [10.6, 17.4]		
Infection probability of one spore (x 10 ⁻³)	1.47 [0.99, 2.19]	1.96 [1.36, 2.80]		
Cumulative spore production (median x 700)	4.0	68.0		
Asymptotic growth rate (median)	1.5	4.5		
<i>A. parthenogenetica</i>		Low spore dose	High spore dose	
Virulence of infections (effects compared to controls) ¹				
Survival time (acceleration factor)	0.44 [0.27, 0.72]	no effect	no effect	
Growth (size difference)	no effect	depends on size class, no overall effect		
Total number of offspring (median) ²	no effect	no effect	no effect	
Clutch type (odds ratio of nauplii vs. cysts)	no effect	no effect	no effect	
Rate of offspring production (offspring/day) ²	no effect	no effect	no effect	
Timing of offspring production ²	shift to early repr. ³	no effect	no effect	
Parasite transmission and fitness				
Spore production rate (spores/mL/5 days x 700)				
- day 15	16.6 [11.1, 24.9]	0.7 [0.4, 1.1]	6.9 [3.1, 15.3]	
- day 30	122.8 [81.3, 185.4]	1.6 [0.9, 2.7]	8.2 [3.6, 18.3]	
- day 45	113.3 [71.2, 179.3]	1.7 [1.0, 3.0]	2.8 [1.2, 6.4]	
- day 60	77.8 [47.6, 127.2]	3.5 [2.0, 6.0]	4.7 [2.0, 11.2]	
Infection probability of one spore (x 10 ⁻³)	2.36 [1.59, 3.52]	0.92 [0.62, 1.37]		
Cumulative spore production (median x 700)	228.0	4.0	24.0	
Asymptotic growth rate (median)	7.2	1.3	2.6	

Notes: ¹ Only significant virulence effects are reported; estimates are based on predictive models and expressed compared to controls. Acceleration factors and odds ratios < 1 indicate respectively a shorter time to, or a lower probability of, the event. The probability of reproduction and time until maturity could not be analyzed for *A. parthenogenetica* because almost all females started reproducing immediately. Virulence analyses were done for infected vs. control individuals, excluding any individuals that died before day 15. ² Results did not differ when nauplii or cysts were given a double weight. ³ See Fig. 3.

either spore dose) did not ($t = 0.7$ and 0.5 , $p = 0.86$ and 0.94 for low and high dose, respectively). Survival was also lower for individuals from the smallest size class ($\chi^2(2) = 11.5$, $p < 0.01$).

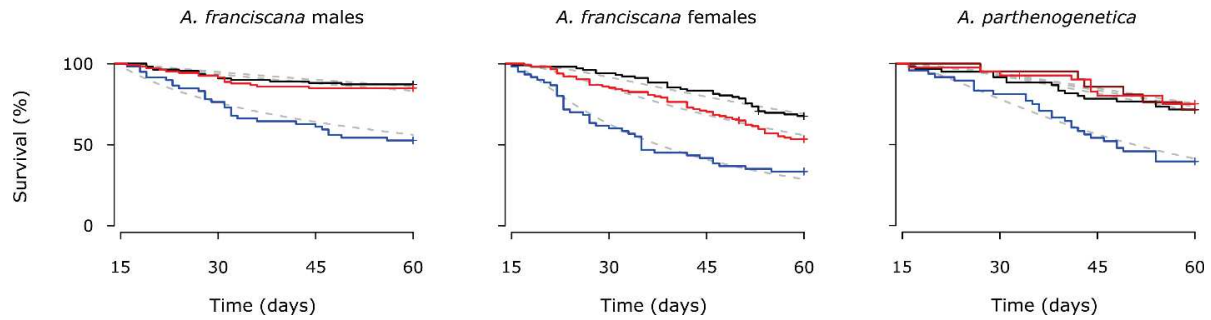


Figure 2. Survival curves for infected individuals (*A. rigaudi*: blue, *E. artemiae*: red, *E. artemiae* high dose: dark red) and control individuals (black). Note that because the analyses had a cutoff at day 15, these graphs neglect all mortality occurring before day 15. The curves shown here are averaged across size class and origin for *A. franciscana* and across size classes in *A. parthenogenetica*. For *A. parthenogenetica*, the aberrant group (Batch 34 ± 2 days old, Size class 7.5 mm) was excluded. Model estimates for each curve are plotted in gray.

More than 90% of host growth occurred between days 1 and 30 (Supp. Table 2), so only this period was analyzed. For *A. franciscana*, growth was significantly lower for males ($\chi^2(1) = 133.5$, $p < 0.0001$) and for individuals from the larger size class ($\chi^2(1) = 49.6$, $p < 0.0001$); but was independent of infection. *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* ($\chi^2(6) = 13.2$, $p = 0.04$). There was no effect of treatment in the absence of the interaction with size class ($\chi^2(3) = 3.0$, $p = 0.39$), but larger size classes grew less ($\chi^2(2) = 33.9$, $p < 0.0001$).

Parasite infection decreased total reproduction in *A. franciscana* females, but had no effect on the timing of reproductive investment (Fig. 3). For *A. franciscana*, the total number of offspring produced was lower when infected with *E. artemiae* than when non-exposed, and much lower when infected with *A. rigaudi* (overall $\chi^2(2) \geq 41.8$, $p < 0.0001$; all treatments significantly different, $p < 0.001$; Fig. 3). When decomposed, several effects became apparent. 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 (effect of infection $\chi^2(2.1) = 23.4$, $p < 0.0001$; post hoc for *A. rigaudi* $t = 4.7$, $p < 0.0001$; 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 ($\chi^2(2) = 31.2$, $p < 0.0001$); this effect was driven by *A. rigaudi* ($z = -5.3$, $p < 0.0001$) and was non-significant for *E. artemiae* ($z = -2.1$, $p = 0.07$). For *A. franciscana* females that did reproduce, infection with *A. rigaudi* increased the proportion of nauplii clutches (effect of infection $\chi^2(2) = 19.1$, $p < 0.0001$; post hoc for *A. rigaudi* $z = 4.1$, $p < 0.0001$; post hoc for *E. artemiae* $z = 0.8$, $p = 0.69$). The rate of offspring production was significantly reduced by infection with *E. artemiae* for all weights of nauplii vs. cysts (effect of infection $\chi^2(2) \geq 8.4$, $p \leq$

0.01; post-hoc for *E. artemiae* $z \geq 2.9$, $p < 0.01$), but was only significantly reduced by infection with *A. rigaudi* when cysts were weighted twice as much as nauplii ($z = -2.4$, $p = 0.04$). Finally, the timing of offspring production was independent of infection status (effect of interaction with time $\chi^2(4) \leq 2.2$, $p \geq 0.69$ depending on the weight of nauplii vs. cysts). Size class was non-significant in all models.

Parasite infection had no effect on the total reproduction of *A. parthenogenetica* females (Fig. 3). The effects of parasite 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 total number of offspring produced, nor the proportion of live clutches, nor the rate of offspring production were affected by infection with either parasite ($\chi^2(3) \leq 5.6$, $p > 0.13$ depending on the weight of nauplii vs. cysts; $\chi^2(2) = 1.4$, $p = 0.71$; $\chi^2(3) \leq 5.8$, $p > 0.12$). Instead, larger females produced more offspring ($\chi^2(2) \geq 8.8$, $p = 0.01$). However, infection with *A. rigaudi* led to a significant shift towards earlier reproduction (Fig. 4; effect of interaction with time $\chi^2(4) \geq 14.1$, $p < 0.03$ depending on the weight of nauplii vs. cysts).

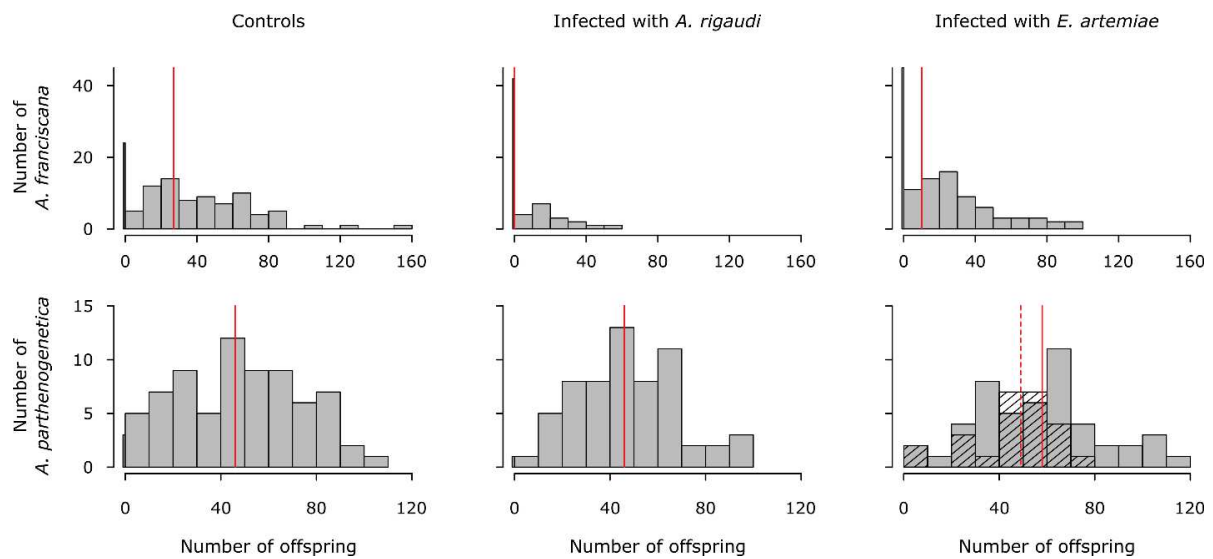


Figure 3. Distributions of the total reproductive output of *A. franciscana* (top) and *A. parthenogenetica* (bottom) females, with medians indicated by the red lines. Weighing the contributions of nauplii and cysts to the total number of offspring generated qualitatively equivalent results; the distributions shown here are for equal weights. Intervals are open on the left, with females producing zero offspring shown in a separate bar. For *A. parthenogenetica* females infected with *E. artemiae*, the hatched distribution and dashed line represent the females exposed to a high spore dose. Note the different scales of the y axes for the two species.

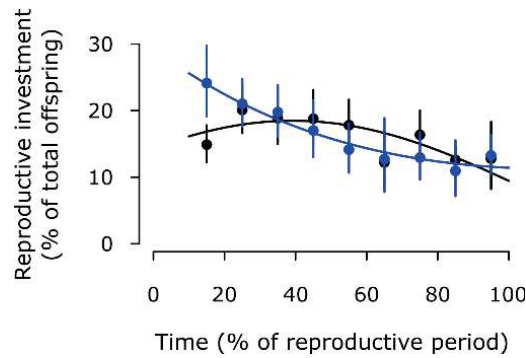


Figure 4. Timing of reproduction in *A. parthenogenetica* controls (black) and infected with *A. rigaudi* (blue). The data is expressed as the size of a given clutch (as a percentage of the female's total offspring production), as a function of the percentage of the reproductive period that has elapsed. 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 equivalent results; the results shown here are for equal weights.

A synopsis of the virulence results is provided in Table **Error! Not a valid bookmark self-reference..**

Table 5. Synopsis of the species level results for the virulence of parasite infections. Two, three, or four '-' indicate moderate, high, or very high costs of infection; '.' indicates no effect. Relevant figures are listed to the right.

	<i>A. rigaudi</i>	<i>E. artemiae</i>	Figures
<i>A. franciscana</i>			
Survival	----	--	Fig. 2
Growth	.	.	
Reproduction	--- ¹	-- ²	Fig. 3
<i>A. parthenogenetica</i>			
Survival	---	.	Fig. 2
Growth	.	.	
Reproduction	. ³	.	Fig. 3, 4

Notes: ¹ Caused by delayed sexual maturity and earlier death. ² Caused by delayed sexual maturity, lower offspring production rate, and earlier death. ³ Earlier death compensated by shift towards early reproduction.

Second, we studied the effects of the host species on the parasite's fitness (summarized in Table 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 rate of spore production varied through time and across host-parasite combinations (significant interaction effect, $\chi^2(3) = 89.8$, $p < 0.0001$, Fig. 5). The rates of spore production were highest in the combinations *A. parthenogenetica*-*A. rigaudi* and *A. franciscana*-*E. artemiae*, and for these combinations the spore production rate peaked on day 30 (generally true on the individual level as well, Supp. Fig. 1). For the other combinations, the rate of spore production increased throughout the experiment (also generally true on the individual level, Supp. Fig. 1). For *A. parthenogenetica* infected with *E. artemiae*, the rate and pattern of spore production

depended on the initial inoculum size (significant interaction effect, $\chi^2(3) = 24.5$, $p < 0.0001$); spore production was notably higher on days 15 and 30 when the initial dose was higher (Fig. 5).

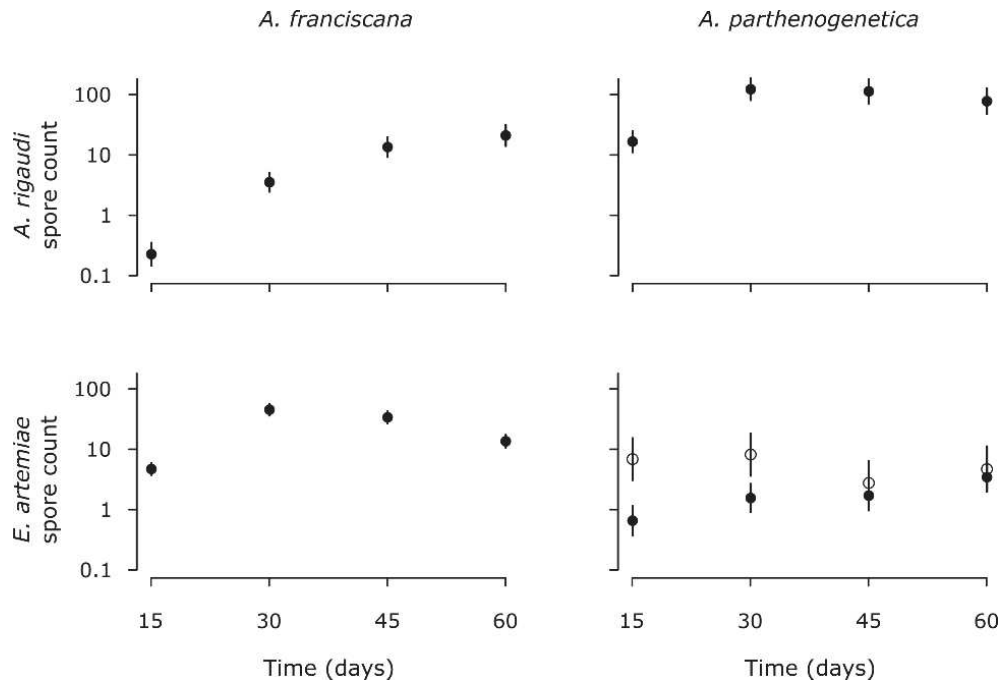


Figure 5. Spore production rates varied through time and across host-parasite combinations. Top: *A. rigaudi*, bottom: *E. artemiae*. The mean spore count at each time is indicated by the points; vertical bars represent the 95% CIs. For *A. parthenogenetica* infected with *E. artemiae*, the low and high spore doses are represented by the solid and empty points, respectively. Spore count is on an \ln scale.

As expected, transmission success increased with the rate of spore production ($\chi^2(1) = 121.6$, $p < 0.0001$, Fig. 6). The slope at which transmission success increased with spore production rate was identical for all host-parasite combinations (no significant effects interacting with *Spore count*), but the intercept varied: *A. parthenogenetica* were slightly more susceptible to *A. rigaudi* and less susceptible to *E. artemiae* than *A. franciscana*, while *A. franciscana* were equally susceptible to both parasites (*Parasite species* and *Host species* interaction, $\chi^2(1) = 170.4$, $p < 0.0001$).

The probability p that a single spore started an infection that could be detected within 7 days, as calculated using the transmission data, is given in Table 4 for each host-parasite combination. *A. rigaudi* was more infectious to *A. parthenogenetica* than to *A. franciscana*, although this difference was not significant ($z = 2.3$, $p = 0.10$); *E. artemiae* was significantly more infectious to *A. franciscana* than to *A. parthenogenetica* ($z = 3.6$, $p < 0.01$).

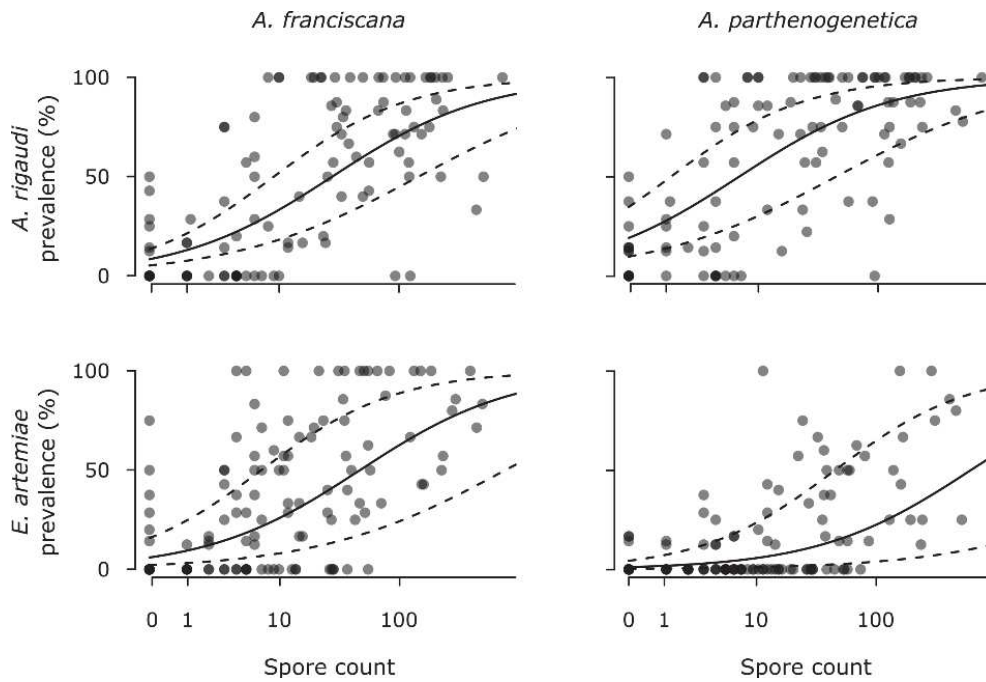


Figure 6. Infectivity in the virulence and transmission experiment. These graphs relate the infection success (percentage of recipients infected) to the spore count in the corresponding spore sample ($\ln + 1$ scale) for *A. rigaudi* (top) and *E. artemiae* (bottom). Note that the graphs are divided by recipient species, not donor species (see Methods). Each point represents a recipient group; overlapping points shade to black. Solid lines are the model predictions; dashed lines illustrate the 95% CIs.

Because the number of spores produced correlated with transmission success for all host-parasite combinations, we were able to use the cumulative spore count and asymptotic growth rate as indicators of parasite fitness. The two measures of overall fitness, cumulative spore count and asymptotic growth rate, were tightly correlated (Fig. 7). The cumulative spore count differed across host-parasite combinations ($\chi^2(4) = 150.2, p < 0.0001$). However, it did not depend on the spore dose for *A. parthenogenetica*-*E. rigaudi* ($p = 0.08$); when the two inoculum sizes for this category were grouped together, all combinations were significantly different except *A. parthenogenetica*-*E. artemiae* and *A. franciscana*-*A. rigaudi* ($p < 0.001, p = 0.79$, respectively). The asymptotic growth rate of an infection was also dependent on the host-parasite combination ($\chi^2(4) = 243.9, p < 0.0001$); all combinations were significantly different except *A. parthenogenetica*-*E. artemiae* (low dose) and *A. franciscana*-*A. rigaudi* ($p < 0.04, p = 0.69$, respectively).

A synopsis of the parasite transmission and fitness results is provided in Table 6.

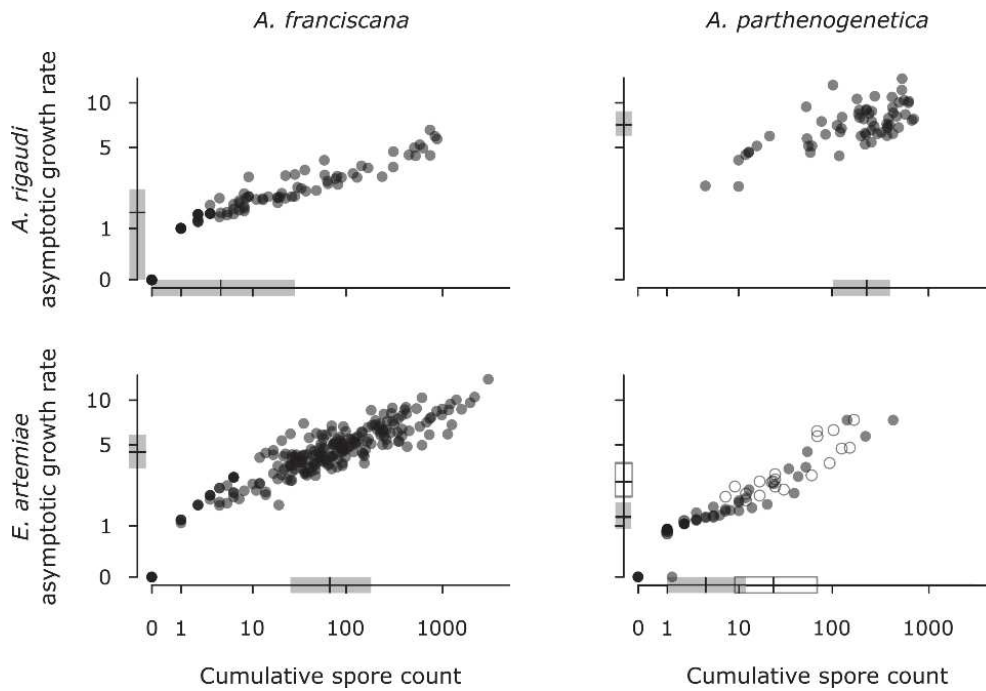


Figure 7. Overall fitness measures of *A. rigaudi* (top) and *E. artemiae* (bottom) infections. The asymptotic growth rate ($\ln + 1$ scale) is shown as a function of the cumulative spore count ($\ln + 1$ scale). The asymptotic growth rate should be maximized during epidemics, while the cumulative spore count, as an estimator of R_0 , should be maximized in endemic conditions (Frank 1996, Hethcote 2000). 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.

Table 6. Synopsis of the species level results for parasite transmission and fitness. One, two, or three ‘+’ indicate low, moderate, or high effect sizes; ‘.’ indicates no effect. Relevant figures are listed to the right.

	<i>A. rigaudi</i>	<i>E. artemiae</i>	Figures
<i>A. franciscana</i>			
Spore production	+	++	Fig. 5
Infection probability of one spore	++	+++	Fig. 6
Fitness	+	++	Fig. 7
<i>A. parthenogenetica</i>			
Spore production	+++	+	Fig. 5
Infection probability of one spore	+++	+	Fig. 6
Fitness	+++	+	Fig. 7

Individual variation in within-host population dynamics

In general, the within-host reproductive rate was independent of the host’s sex or size class (Supp. Table 3). In the combination *A. franciscana*-*A. rigaudi*, males had higher within-host reproductive rates ($\Delta\text{AICc} \geq 10.4$); for *A. parthenogenetica*-*E. artemiae*, within-host reproductive rate was higher for individuals exposed to high initial spore doses ($\Delta\text{AICc} = 13.7$).

In most of the host-parasite combinations, within-host reproductive rate, peak spore count, and peak time were tightly correlated (Fig. 8, Supp. Table 4). Higher reproductive rates were associated with higher peaks in all combinations, although the quantitative effect was small for

A. parthenogenetica infected with *A. rigaudi* (left column, Fig. 8). Higher reproductive rates were also associated with earlier peaks for *A. franciscana* infected with *E. artemiae* and for *A. parthenogenetica* infected with *A. rigaudi*. This correlation was reversed for *A. franciscana* infected with *A. rigaudi*, and absent for *A. parthenogenetica* infected with *E. artemiae* (though note that not many infections in these combinations reached a peak) (right column, Fig. 8).

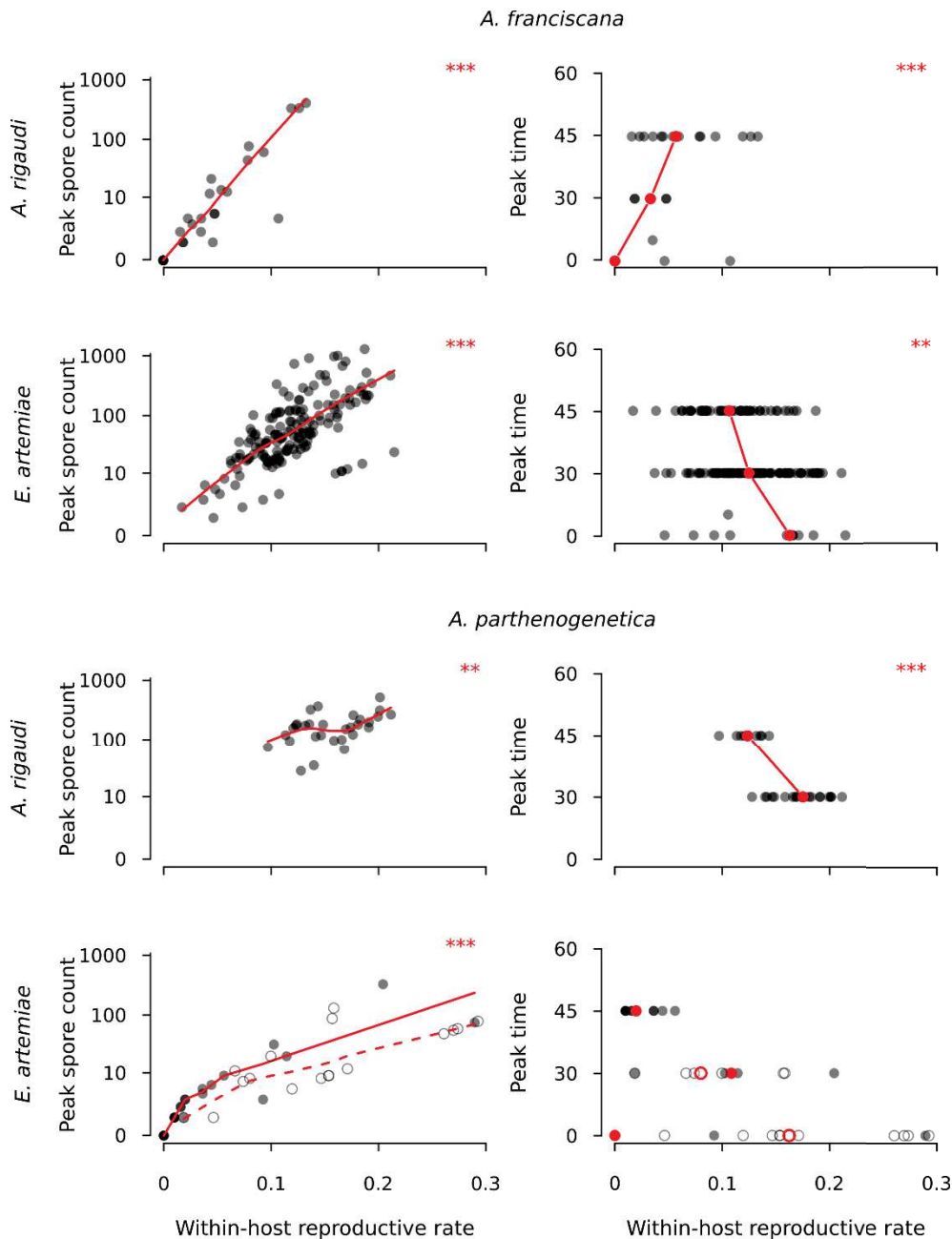


Figure 8. Correlations between within-host reproductive rate, peak spore count, and peak time for every host-parasite combination. Top rows: *A. franciscana* infected with *A. rigaudi* and *E. artemiae*. Bottom rows: *A. parthenogenetica* infected with *A. rigaudi*, *E. artemiae* (solid points and lines: low spore dose; empty points and lines: high spore dose). Only individuals for whom we recorded a true peak are shown. These were used to calculate: 1) correlations, whose level of significance is shown by the asterisks in red (**p < 0.01, ***p < 0.001, Supp. Table 4); 2) median values (for the right column), represented by red circles and connected to show

significant correlations; 3) LOESS fits (in the left column), represented by a red line. Overlapping points shade to black.

Finally, in the combinations *A. parthenogenetica*-*A. rigaudi* and *A. franciscana*-*E. artemiae*, the parasite's within-host reproduction was associated with both survival and reproductive costs to the host. In the first combination, a higher within-host reproductive rate was associated with higher mortality (Fig. 9, $\Delta\text{AICc} = 4.6$, Supp. Table 5). In the second, the association between mortality and parasite reproductive rate was dependent on the host's sex and size class ($\Delta\text{AICc} = 9.7$, Supp. Table 5). There was no significant correlation between the parasite's reproductive rate and the overall reproductive success of female hosts in either of the combinations (Supp. Table 6), nor did the reproductive rate affect the time until maturity or the probability of producing a clutch (Supp. Table 7). However, for *A. franciscana* infected with *E. artemiae*, a high within-host reproductive rate of the parasite was associated with a lower rate of offspring production for the host, in particular for larger females (Fig. 9, $\Delta\text{AICc} \geq 1.4$, Supp. Table 7).

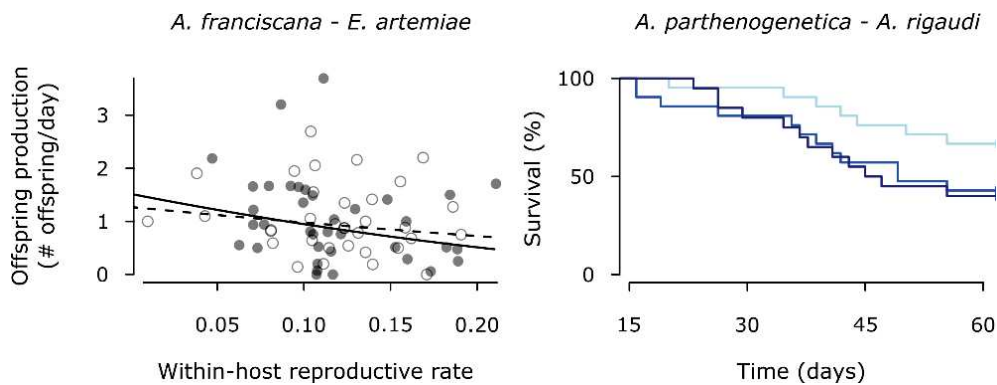


Figure 9. Relationship between virulence and within-host reproductive rate for *A. franciscana* females infected with *E. artemiae* (left) and *A. parthenogenetica* infected with *A. rigaudi* (right). In the offspring production plot, the dashed curves and open circles represent females of size class 4.5; solid curves and circles represent females of size class 5.0. Shown here are the results when nauplii and cysts are given an equal weight. In the survival plot, curves were plotted by dividing hosts into terciles based on within-host reproductive rate (highest rate: darkly colored, lowest rate: light color).

Individual variation in resistance

Among the individuals that survived until we could be certain of their infection status, the rate of resistance varied between 0 and 36% in the different host-parasite combinations (Table 7). For *A. franciscana*, significantly more individuals resisted infection with *A. rigaudi* than with *E. artemiae* ($\chi^2(1) = 10.4$, $p < 0.01$), and this effect was independent of sex ($\chi^2(1) = 0.3$, $p = 0.58$). In contrast, significantly more *A. parthenogenetica* resisted infection with *E. artemiae* ($\chi^2(1) = 20.6$, $p < 0.0001$), with a marginally non-significant difference between the two spore doses ($\chi^2(1) = 3.8$, $p = 0.052$). 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’, so we continued our analyses with these combinations.

Table 7. Number of exposed individuals that were infected or resistant to *A. rigaudi* and *E. artemiae*. These counts excluded all individuals who died before infection status could be definitively determined, i.e. those that died before day 15 of the experiment.

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

For both *A. franciscana* exposed to *A. rigaudi* and *A. parthenogenetica* exposed to a low spore dose of *E. artemiae*, resistant individuals died more quickly than infected individuals (Fig. 10; ΔAICc respectively > 4.5 and $= 1.7$, Supp. Table 8). For *A. franciscana*, resistant males had a higher mortality than resistant females (Fig. 10; $\Delta\text{AICc} > 1.8$, Supp. Table 8).

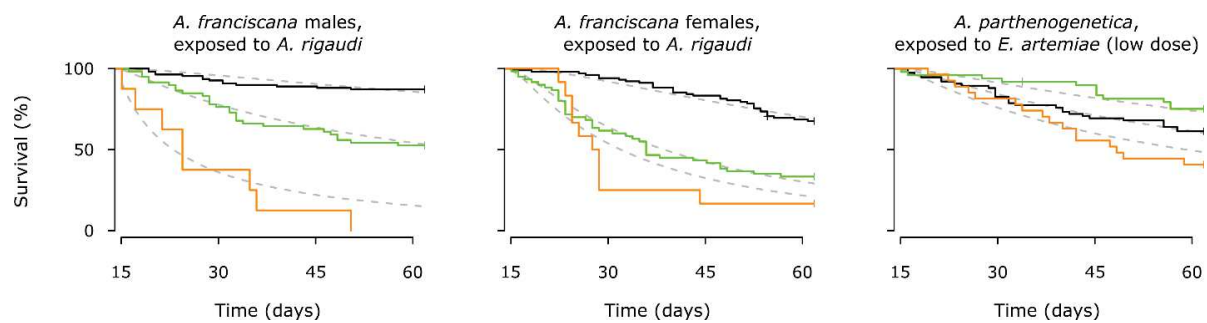


Figure 10. Survival curves for resistant (orange), infected (green), and control (black) individuals. Note that because the analyses had a cutoff at day 15, these graphs neglect all mortality occurring before day 15. The curves shown here are averaged across size class and origin for *A. franciscana* and across size classes in *A. parthenogenetica*. Model estimates for each curve are plotted in gray.

Finally, there was little support for an effect of resistance on reproduction in females of either host species (Supp. Table 9). *A. franciscana* females that resisted infection with *A. rigaudi* behaved similarly to females that became infected (strong effects of *Resistant-Infected-Control*, but no or weak support for a difference between resistant and infected females, Supp. Tables 9 & 10). The analyses suggested that resistant *A. franciscana* that did reproduce had a higher offspring production rate than both control and infected females ($\Delta\text{AICc} \geq 2.7$, Supp. Table 9), but this effect was based on a very restricted subset of resistant-and-reproducing individuals ($n = 2$). Even assuming that this effect was not spurious, it could not compensate for the loss of reproductive success caused by the shorter reproductive period and reduced probability of producing a clutch. The reproductive behavior of *A. parthenogenetica* females that resisted infection with a low dose of *E. artemiae* was similar to that of infected and control females (Supp. Tables 9 & 10).

DISCUSSION

We infected two species of brine shrimp, *A. franciscana* and *A. parthenogenetica*, with one of two sympatric microsporidian gut parasites, *A. rigaudi* and *E. artemiae*, and tracked the life history of both hosts and parasites. A brief synopsis of the results is shown in Table 8. Because the experiments were done separately for *A. franciscana* and *A. parthenogenetica*, the results were presented largely from the point of view of the host (i.e. a horizontal reading of Table 8). We now shift to a parasite viewpoint (i.e. a vertical reading of Table 8) to discuss the life histories and virulence of each parasite, the costs of host resistance, and the overall patterns of multi-host virulence and transmission.

Table 8. Qualitative synopsis of results.

Host species	Parasite species	
	<i>A. rigaudi</i>	<i>E. artemiae</i>
<i>A. franciscana</i>	moderately infectious highly virulent low parasite transmission ⇒ low parasite fitness, mismatched host & parasite	highly infectious moderately virulent high parasite transmission ⇒ high parasite fitness, matched host & parasite
<i>A. parthenogenetica</i>	highly infectious moderately virulent high parasite transmission ⇒ high parasite fitness, matched host & parasite	poorly infectious avirulent low parasite transmission ⇒ low parasite fitness, mismatched host & parasite

Before discussing our results, we note an important caveat of this study: our experiments were restricted to single infections, while coinfections of *A. rigaudi* and *E. artemiae* are common in the field (Lievens et al. in prep.a). Coinfection could have significant consequences for the evolution of the microsporidians' life history and the *Artemia*'s responses (Rigaud et al. 2010, Alizon et al. 2013); by failing to take it into account, we risk misinterpreting the parasite exploitation and host defense results. Future work will determine whether this has been the case.

Life history of A. rigaudi

A. rigaudi was able to infect and produce transmission stages in both *Artemia* species, but it was much more successful when infecting *A. parthenogenetica*: *A. rigaudi* infections produced roughly 57 times as many spores in the latter host compared to the former, reaching an asymptotic growth rate that was 4.8 times as high (Table 4).

A. rigaudi spores were half again as infectious to *A. parthenogenetica* as they were to *A. franciscana* (Fig. 1, Table 4). Previous experiments failed to detect this difference, presumably because the uncontrolled inoculum size produced saturated levels of infection (Lievens et al. in prep.a). Indeed, the present result explains the discrepancy between Lievens et al.'s lab results

and the consistent association of *A. rigaudi* with *A. parthenogenetica* in natural populations (Lievens et al. in prep.a, Rode et al. 2013c).

Finally, *A. rigaudi* infections were characterized by substantial virulence. Infection halved the expected lifespan of *A. parthenogenetica*, and reduced that of male and female *A. franciscana* by about two thirds (Fig. 2). For *A. parthenogenetica*, females infected with *A. rigaudi* maintained the same lifetime reproductive success as control females, despite having shortened lifespans (Fig. 3, Table 4); they accomplished this by shifting towards earlier reproduction (fecundity compensation, e.g. Minchella and Loverde 1981, Agnew et al. 2000, Chadwick and Little 2005) (Fig. 4). For *A. franciscana* females, in contrast, infection with *A. rigaudi* entailed severe reproductive costs (median of 0 offspring vs. 27 in controls, Fig. 3, Table 4). This reduction was caused by delayed sexual maturity, which in combination with their accelerated mortality reduced their probability of producing a clutch. Interestingly, females that did reproduce had the same rate and timing of offspring production as controls, but were four times more likely to produce clutches of live young. Considering that *Artemia* generally produce cysts when stressed (Clegg and Trotman 2002), this result seems counterintuitive. One intriguing possibility is that a shift towards live born offspring is advantageous for the host: if infected mothers can provide transgenerational immune priming to their offspring, it may be more worthwhile to produce protected nauplii, which could have a competitive advantage in the parasite-infested environment, than to produce protected cysts, whose hatching environment is unknown (Norouzitallab et al. 2015).

Life history of E. artemiae

Like *A. rigaudi*, *E. artemiae* was much more successful in one host, in its case *A. franciscana*. For a given inoculum size, *E. artemiae* infections produced 17 times as many spores in *A. franciscana* as they did in *A. parthenogenetica*, and their asymptotic growth rate was 3.5 times as high (Table 4).

E. artemiae spores were also twice as infectious to *A. franciscana* as they were to *A. parthenogenetica*, confirming previous lab and field results (Fig. 1, Table 4) (Lievens et al. in prep.a, Rode et al. 2013c).

Finally, while *E. artemiae* infections in *A. parthenogenetica* were completely avirulent, the parasite did display virulence when infecting *A. franciscana*. The life expectancy of both males and females was reduced by roughly one third when infected, as was the rate of offspring production for reproducing females (Fig. 2, Table 4). These effects, in conjunction with a delay in sexual maturity, significantly reduced the lifetime reproductive success of infected *A. franciscana* females (median of 10 offspring vs. 27 in controls, Fig. 3, Table 4).

Multihost parasites and their match & mismatch hosts

In our two-host, two-parasite system, each of the parasites performed well on a different host species: *A. rigaudi* was very successful in *A. parthenogenetica*, while *E. artemiae* performed well in *A. franciscana*. In the following discussion, we will refer to these as the ‘matched’ combinations. The reversed combinations also produced viable transmission stages, but at much lower rates; we will call these the ‘mismatched’ combinations.

Our identification of matched vs. mismatched combinations corresponds well with the published field data for this system, which shows that *A. rigaudi* and *E. artemiae* are always more prevalent in respectively *A. parthenogenetica* and *A. franciscana* (Lievens et al. in prep.a, Rode et al. 2013c). Furthermore, Lievens et al. showed that *A. rigaudi* is dependent on its matched host to persist in the natural host community, and suggested that the same is true for *E. artemiae*. As *E. artemiae* and *A. rigaudi* performed roughly equally poorly in their mismatched hosts, this experiment supports that suggestion.

Based on the field and experimental data, we expect the selection pressures in the natural *Artemia*-microsporidian community to be asymmetrical between hosts & parasites. The parasites should only coevolve with their matched hosts: their reliance on the matched hosts as reservoirs will create source-sink dynamics in the host community, preventing them from adapting to the mismatched hosts (Lenormand 2002, Dobson 2004, Viana et al. 2014). The reverse, however, should not be true. *A. franciscana* and *A. parthenogenetica* are regularly exposed to their mismatched parasites (Lievens et al. in prep.a), so we expect them to be under selection to resist or tolerate both microsporidians (Antonovics et al. 2013).

Parasite life history and host ecology in matched combinations

A. rigaudi was more virulent than *E. artemiae* in their respective matched hosts, and indeed in both host species. This result confirms Rode et al. (2013c)’s observational conclusion that infection with *A. rigaudi* had a larger impact on hosts than infection with *E. artemiae*. Interestingly, Rode et al. found that sexually mature females were less likely to be brooding a clutch when they were small and infected with *A. rigaudi*, while in our study *A. rigaudi* did not affect the frequency of clutching once sexual maturity had been reached (data not shown). The different conditions in the field may be responsible for this seemingly additional virulence (e.g. food limitation, Brown et al. 2000, Bedhomme et al. 2004, Vale et al. 2011; temperature, Mitchell et al. 2005, Vale et al. 2008; other stressors, Gleichsner et al. 2016). The virulence induced by *A. rigaudi* and *E. artemiae* also had different characteristics in the matched hosts: *A. rigaudi* induced survival virulence in *A. parthenogenetica*, who alleviated the resulting reproductive cost by shifting towards earlier reproduction, while *E. artemiae* reduced both the survival and the reproduction of *A. franciscana*. This difference could indicate that *A. rigaudi* and *E. artemiae* exploit different host resources (general resource depletion, e.g. Rivero et al. 2007, vs. specific appropriation of reproductive resources, e.g. Hurd 1998).

In addition to being more virulent, *A. rigaudi* produced spores at a much higher rate than *E. artemiae* when each infected its matched host. The higher spore production rate of *A. rigaudi* was not compensated by a loss of infectiousness; on the contrary, *A. rigaudi* and *E. artemiae* spores were similarly infective to their matched hosts. However, it should be noted that we do not know how long the spores can persist in the environment, a factor which has substantial influence on the success of parasites with free-living transmission stages (Alizon and Michalakis 2015).

All else being equal, the higher virulence and transmission rates of *A. rigaudi* compared to *E. artemiae* suggest that *A. rigaudi* has been selected for high rates of exploitation and virulence, while *E. artemiae* has been selected for lower exploitation and virulence. Many possible factors could underlie this difference; we discuss two of them. First, it is well known that epidemic conditions tend to select for higher parasite virulence than endemic conditions (Frank 1996). In the field, *A. rigaudi* has seasonally epidemic patterns, increasing in prevalence from 0 to up to 100% as the proportion of *A. parthenogenetica* in the population increases, while *E. artemiae* is more generally present (Lievens et al. in prep.a). Thus, it is possible that the seasonality of its matched host (*A. parthenogenetica*) has imposed epidemic conditions on *A. rigaudi*, selecting for fast exploitation and high virulence. A second possibility is that the multi-parasite context in which *A. rigaudi* normally operates has selected for a high rate of exploitation. *A. parthenogenetica*, its matched host, is more often coinfecting by both microsporidians than *A. franciscana*, the matched host of *E. artemiae* (Lievens et al. in prep.a). In addition, *A. parthenogenetica* is an intermediate host for several cestodes, notably *Flamingolepis liguloides*, which can reach prevalences of up to 100% in summer (Sánchez et al. 2012, E.J.P. Lievens unpublished data). *F. liguloides* castrates its host, prolongs its lifespan, and causes it to store more lipids (Amat et al. 1991a, Sánchez et al. 2012). Because *A. rigaudi* and *F. liguloides* often share a host (Rode et al. 2013b), *A. rigaudi* may have evolved higher virulence to compete against its competitors' drain on host resources (Graham 2008, Alizon et al. 2013). In contrast, *A. franciscana* has few cestode parasites in its invasive range (Sánchez et al. 2012).

Parasite virulence in mismatched combinations

Several theoretical predictions have been made for the evolution of parasite virulence in multi-host parasites with source-sink population dynamics, all of which agree that virulence should depend exclusively on the optimum in the source host (Regoes et al. 2000, Woolhouse et al. 2001, Dobson 2004, Gandon 2004). Predictions of virulence in the sink 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 sink 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 sink 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 sink 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 sink 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 1996, Jaenike and Dombeck 1998, Perlman and Jaenike 2003) and for the *Drosophila* C virus (Longdon et al. 2015), which exhibit a range of exploitation and correlated virulence across host species.

Overall, our results provide varying support for the theoretical predictions of virulence evolution in multi-host parasites: in one case, we appear to be dealing with decoupled, 'runaway' virulence, while in the second the differences in virulence may be driven by levels of host resistance. In-depth studies of more naturally occurring multi-host systems should help indicate whether either, or both, of these patterns is a common outcome.

Within-host population dynamics in matched & mismatched combinations

In both matched combinations, the rate of spore production peaked around day 30 (on average, Fig. 5; for individuals, Supp. Fig. 1). Because the rate of spore production of *A. rigaudi* and *E. artemiae* is a good indicator of parasite burden (see Methods), this indicates that the within-host microsporidian populations are under density-dependent regulation. In parasites, density dependence can be caused by an exhaustion of host resources such as energy and space (e.g. Selvan et al. 1993, Ebert et al. 2000, Luong et al. 2011, Rynkiewicz et al. 2015), by the hosts' immune activity (e.g. Paterson and Viney 2002), or both (Tyler et al. 2001, Luong et al. 2011). Distinguishing between resource-driven and immune-mediated density dependence is difficult (cf. Keymer 1982, Pollitt et al. 2013), but the relationships between within-host reproductive rate, peak spore count, and peak time allow us to elaborate on the different possibilities. If density dependence is caused by a depletion of host resources, the peak spore counts should be

independent of the within-host reproductive rate (the peak is expected to occur when resources have been depleted, irrespective of the rate at which they were consumed). Instead, a higher reproductive rate should be associated with an earlier peak, because the finite resource becomes exhausted more quickly. These predictions correspond to the within-host dynamics of *A. rigaudi* in *A. parthenogenetica*, but clearly not to those of *E. artemiae* infecting *A. franciscana*. For the latter, higher reproductive rates were associated with higher peaks (Fig. 8), implying that resource depletion cannot be the (only) source of density-dependent regulation. In this case, the parasite population could be limited by the host's immune system, with rapidly reproducing parasites better able to 'outrun' an immune response with delayed or density-dependent activation (Alizon and van Baalen 2008).

In the mismatched combinations, within-host reproductive rates were lower, and parasite populations rarely peaked (Fig. 8). In *A. franciscana* infected with *A. rigaudi*, the average rate of spore production did appear to saturate towards the end of the experiment, reaching comparable peak spore counts to those of *E. artemiae* in the same host (Fig. 5). This could indicate that *A. rigaudi* and *E. artemiae* are subject to the same density-dependent mechanisms in *A. franciscana*. In *A. parthenogenetica* infected with *E. artemiae*, the rate of spore production was more or less stable for hosts infected with a high spore dose; those infected with a low spore dose produced fewer spores at first, but caught up towards the end of the experiment (Fig. 5). We interpret this as indicating that density-dependent regulation imposes a maximal rate of spore production, and that this rate is reached more quickly when the initial parasite density is higher (Castaneda et al. 1987, Ebert et al. 2000). Interestingly, the peak spore counts for *A. rigaudi* and *E. artemiae* in *A. parthenogenetica* differed by an order of magnitude, suggesting that different density-dependent mechanisms act on the two parasites.

Resistance in matched & mismatched combinations

In the matched host-parasite combinations, uninfected individuals were rare or nonexistent (Table 7), and suffered no detectable survival cost (data not shown). It is possible that an extremely high mortality rate of resistant individuals caused them to die before we could reliably detect infection, leading us to underestimate both the frequency and the cost of resistance. However, survival rates for the matched combinations were universally high in the infectivity experiment, which lasted one week. Any mortality conferred by resistance would therefore have to be incurred precisely in the second week of infection, which is unlikely. It is more probable that the high rates of infection reflect selection on the parasite to evade or overcome resistance in its matched host (Hasu et al. 2009).

In the mismatched host-parasite combinations, however, up to one third of the exposed hosts were uninfected (Table 7). We call these individuals resistant because their life histories differed clearly from those of control or infected hosts (discussed below), suggesting that their lack of infection was the result of an active mechanism.

Resistance had dramatic impacts on the fitness of mismatched hosts. Resistant individuals died more rapidly than both infected and control hosts, with no detectable compensation through increased fecundity (Fig. 10). Because the parasite was absent, the cost of resistance must have been induced by the host itself, as a consequence of its immune reaction upon exposure (Schmid-Hempel 2003, Graham et al. 2005). Similarly disproportionate costs of resistance, with uninfected hosts dying more rapidly than even infected hosts, have been found in e.g. *Daphnia* resisting the bacterium *Pasteuria* (Little and Killick 2007, though see Labbé et al. 2010), and naïve isopods resisting infection with a helminth (Hasu et al. 2009).

The extreme cost of resistance in mismatched host-parasite combinations is intriguing. Although resistance is sometimes less costly for hosts that coevolve with a parasite than for naïve hosts (Moret 2003, Hasu et al. 2009, Auld et al. 2013), the mismatched hosts in our system are not naïve: *A. franciscana* and *A. parthenogenetica* are regularly exposed to both *A. rigaudi* and *E. artemiae* (Lievens et al. in prep.a). Furthermore, host resistance has been shown to evolve quickly in a similar host-parasite system (*Daphnia magna*-*Octosporea bayeri*, Zbinden et al. 2008). A possible explanation for the existence of this maladaptive host response is that the source-sink dynamics acting in the host-parasite community prevent the parasite from evolving to reduce its impact on the mismatched host. In turn, selection on the host to reduce its response to the mismatched parasite could be countered by other factors, such as the need to maintain its overall immune capacity (Graham et al. 2005).

Optimal host exploitation in matched combinations?

Much of the theory of parasite evolution is based on the assumption that increasing host exploitation both promotes transmission, and shortens the infectious period by inducing host death or immune clearance. Non-linear relationships between these components are then expected to select for optimal levels of exploitation (Gandon 2004, Alizon et al. 2009). In our experiment, we measured host exploitation as the within-host reproductive rate. We can therefore ask whether parasite reproduction was related to virulence and transmission in the coevolving (i.e. the matched) host-parasite combinations, and whether certain levels of host exploitation optimized parasite fitness.

In *A. franciscana* infected with *E. artemiae*, increasing rates of host exploitation led to higher peak spore counts (Fig. 8), but did not affect host mortality in any robust way. As a result, the highest parasite fitness was attained by infections with maximal rates of within-host reproduction (Fig. 11). In *A. parthenogenetica* infected with *A. rigaudi*, host exploitation had a more subtle effect on peak spore production, influencing its timing much more than its extent (Fig. 8). Higher within-host reproductive rates were therefore associated with higher asymptotic growth rates, which are influenced by the timing of transmission, but not with higher cumulative spore counts, which are not (Fig. 11). The additional host mortality associated with high rates of host exploitation could not reverse this pattern (Fig. 9).

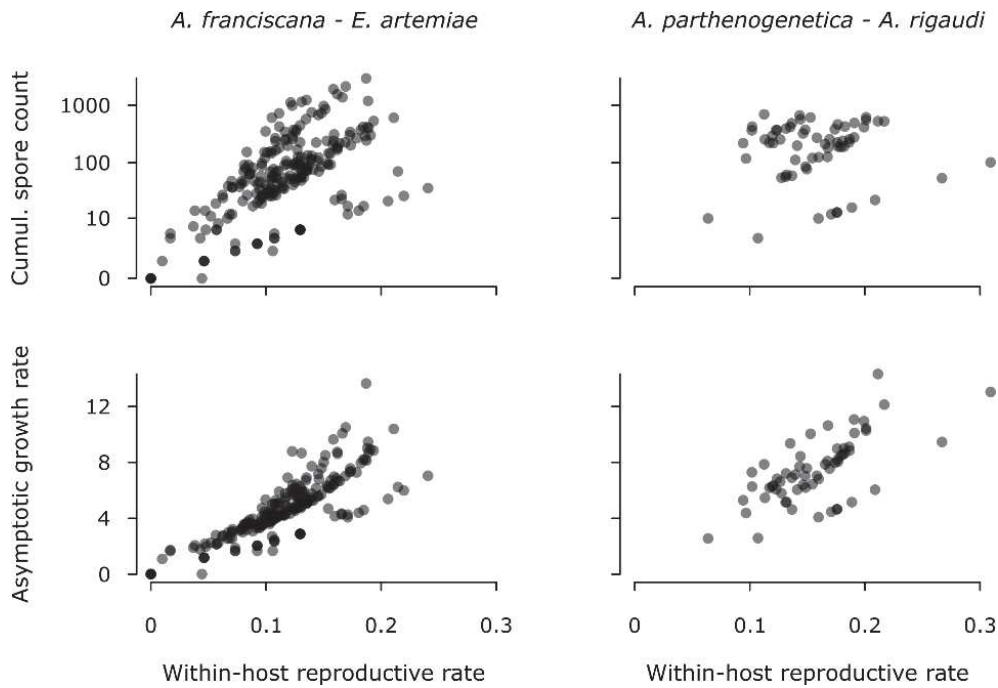


Figure 11. Fitness as a function of host exploitation for the matched host-parasite combinations. Shown are the cumulative spore counts (top, $\ln + 1$ scale) and asymptotic growth rates (bottom) for *A. franciscana* hosts infected with *E. artemiae* (left) and *A. parthenogenetica* hosts infected with *A. rigaudi* (right), as a function of the within-host reproductive rate. Each point represents one infected host; overlapping points shade to black.

Based on our results, therefore, both *A. rigaudi* and *E. artemiae* should generally be selected for higher rates of host exploitation. This may well be the case (Ebert 1998). However, there are several probable alternatives. First, we have ignored an important element of microsporidian fitness: spore quality. It is not implausible that a trade-off exists between the rate of spore production (spore quantity) and spore infectiousness or longevity (spore quality; Alizon and Michalakakis 2015, though see Walther and Ewald 2004), in which case our estimates of parasite fitness would be inflated at the far end of the exploitation scale. Second, it is possible that parasite genotypes with extremely high reproductive rates killed their hosts before we could reliably detect infection, causing a drop in parasite fitness beyond our recorded exploitation range. This is particularly plausible for *A. rigaudi* (Fig. 9). Finally, the effects of within-host reproductive rate could have been obscured by variation in host quality (a ‘big house, big car’ effect), or by variation in the genetic diversity of infections (via within-host competition, Rigaud et al. 2010). An experiment with explicit control of host and parasite genotypes would be necessary to investigate these possibilities.

CONCLUSION AND PERSPECTIVES

In nature, multi-host parasites and multi-parasite hosts are likely to be the rule, rather than the exception (Cleaveland et al. 2001, Taylor et al. 2001, Streicker et al. 2013). Empirically, however, we know little about the interplay between parasite fitness, virulence, and infectivity

in these complex systems (Rutrecht and Brown 2009, Rigaud et al. 2010). In this study, we quantified infectivity, virulence, and parasite life history in all combinations of a naturally occurring two-host, two-parasite system. We showed that both parasites, *A. rigaudi* and *E. artemiae*, perform well on one of the two hosts. The exploitation strategies employed by the two microsporidians in their specialized hosts, as manifested by the virulence and within-host growth, appear to be quite different. This specialization is in turn associated with suboptimal exploitation, aberrant virulence, and the induction of large costs of resistance in the non-specialized host. Our study represents a rare empirical contribution to the study of parasite evolution in multi-host systems, highlighting in particular the difficulties faced by parasites adapting to multiple host species.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Host survival during the infectivity experiment.

Parasite species	Dose (spores/individual)	Nb. exposed	Nb. died	% Survived
<i>A. franciscana</i>				
Controls	0	17	7	59%
<i>A. rigaudi</i>	400	20	3	85%
	800	20	3	85%
	1600	20	1	95%
	3200	20	6	70%
	6400	20	4	80%
<i>E. artemiae</i>	400	20	10	50%
	800	20	4	80%
	1600	20	9	55%
	3200	20	1	95%
	6400	20	8	60%
<i>A. parthenogenetica</i>				
Controls	0	4	0	100%
<i>A. rigaudi</i>	400	16	0	100%
	800	20	1	95%
	1600	20	0	100%
	3200	8	0	100%
	6400	4	0	100%
<i>E. artemiae</i>	400	20	2	90%
	800	20	0	100%
	1600	20	1	95%
	3200	20	2	90%
	6400	20	2	90%

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

Hosts	Mean difference	<i>p</i> (mean difference ≠ 0)
<i>A. franciscana</i> males		
Growth between days 1 & 30	2.1	< 0.0001
Growth between days 30 & 60	0	0.44
<i>A. franciscana</i> females		
Growth between days 1 & 30	2.8	< 0.0001
Growth between days 30 & 60	0.3	< 0.0001
<i>A. parthenogenetica</i>		
Growth between days 1 & 30	1.8	< 0.0001
Growth between days 30 & 60	0.2	< 0.0001

Supplementary Table 3. Model comparison: experimental variables explaining the variation in within-host reproductive rate. Models were run separately for every host-parasite combination; full models also included a random effect of *Origin* (for *A. franciscana*) or *Batch* (for *A. parthenogenetica*). *w* is the Akaike weight of each model.

Host-parasite combination	AICc	ΔAICc	<i>w</i>
<i>A. franciscana</i> infected with <i>A. rigaudi</i>			
~ Sex	-171.7	0	0.98
~ Sex + Size class	-163.6	8.1	0.02
~ 1	-161.3	10.4	0.01
~ Sex + Size class + Sex : Size class	-156.9	14.8	0.00
~ Size class	-153.3	18.4	0.00
<i>A. franciscana</i> infected with <i>E. artemiae</i>			
~ 1	-747.0	0	0.98
~ Sex	-737.8	9.2	0.01
~ Size class	-737.8	9.2	0.013
~ Sex + Size class	-728.6	18.4	0.00
~ Sex + Size class + Sex : Size class	-721.8	25.2	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>			
~ 1	-179.9	0	1.00
~ Size class	-162.4	17.5	0.00
<i>A. parthenogenetica</i> infected with <i>E. artemiae</i>			
~ Spore dose	-88.9	0	1.00
~ 1	-75.2	13.7	0.00
~ Spore dose + Size class	-74.7	14.2	0.00
~ Spore dose + Size class + Spore dose : Size class	-64.2	24.7	0.00
~ Size class	-61.3	27.6	0.00

Supplementary Table 4. Correlations between within-host reproductive rate, peak spore count, and peak time within each host-parasite combination. Only individuals for whom a peak could be observed were included in these analyses (thereby excluding individuals whose highest spore count was right before their death). *p*-values adjusted for multiple testing using Holm's correction.

Host-parasite combination	Spearman's ρ	<i>p</i>	adjusted <i>p</i>
<i>A. franciscana</i> infected with <i>A. rigaudi</i>			
Within-host reproductive rate & peak spore count	0.83	< 0.0001	< 0.0001
Within-host reproductive rate & peak time	0.93	< 0.0001	< 0.0001
Peak time & peak spore count	0.62	< 0.001	< 0.001
<i>A. franciscana</i> infected with <i>E. artemiae</i>			
Within-host reproductive rate & peak spore count	0.41	< 0.0001	< 0.0001
Within-host reproductive rate & peak time	0.65	< 0.0001	< 0.0001
Peak time & peak spore count	-0.25	< 0.01	< 0.01
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>			
Within-host reproductive rate & peak spore count	0.07	0.73	0.73
Within-host reproductive rate & peak time	0.50	< 0.01	0.01
Peak time & peak spore count	-0.77	< 0.0001	< 0.0001
<i>A. parthenogenetica</i> infected with <i>E. artemiae</i> – low spore dose			
Within-host reproductive rate & peak spore count	0.21	0.36	0.72
Within-host reproductive rate & peak time	0.97	< 0.0001	< 0.0001
Peak time & peak spore count	0.08	0.75	0.75
<i>A. parthenogenetica</i> infected with <i>E. artemiae</i> – high spore dose			
Within-host reproductive rate & peak spore count	0.02	0.93	0.93
Within-host reproductive rate & peak time	0.79	< 0.001	< 0.001
Peak time & peak spore count	-0.51	0.04	0.07

Supplementary Table 5. Model comparison: link between survival and within-host reproductive rate. For each host-parasite combination, *Reproductive rate* was allowed to interact with all of the experimentally manipulated factors (the base model). The base model for *A. franciscana* included *Sex***Size class* and a frailty component for *Origin* (lognormal distribution, see Results). The base model for *A. parthenogenetica* included *Size class* and a frailty component for *Batch* (log-logistic distribution, see Results). Note that these analyses only included individuals that survived until at least day 15, when infection status could be definitively determined and for whom we could measure a reproductive rate. *w* is the Akaike weight of each model.

Host-parasite combination	AICc	Δ AICc	<i>w</i>
<i>A. franciscana</i> infected with <i>E. artemiae</i>			
Base model + Reproductive rate + Reproductive rate : Sex + Reproductive rate : Size class + Reproductive rate : Sex : Size class	738.6	0.0	0.99
Base model + Reproductive rate + Reproductive rate : Size class	748.3	9.7	0.01
Base model + Reproductive rate + Reproductive rate : Sex + Reproductive rate : Size class	750.1	11.5	0.00
Base model	751.5	12.9	0.00
Base model + Reproductive rate	752.6	13.9	0.00
Base model + Reproductive rate + Reproductive rate : Sex	754.4	15.7	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>			
Base model + Reproductive rate	314.0	0.0	0.87
Base model + Reproductive rate + Reproductive rate : Size class	318.6	4.6	0.09
Base model	319.9	5.8	0.05

Supplementary Table 6. Overall tests: link between total number of offspring produced and within-host reproductive rate.

Host-parasite combination	ρ	p
<i>A. franciscana</i> infected with <i>E. artemiae</i>*		
Spearman's ρ correlation	0.01	0.89
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>*		
Spearman's ρ correlation	-0.22	0.09

*Shown for the tests that weighted nauplii and cysts equally; giving either offspring type a double weight produces qualitatively equivalent results.

Supplementary Table 7. Model comparison: link between reproduction and within-host reproductive rate. For each host-parasite combination, *Reproductive rate* was allowed to interact with all of the experimentally manipulated factors (the base model). The base model for *A. franciscana* included *Size class* and *Origin* as a random or frailty component; the base model for *A. parthenogenetica* included *Size class* and *Batch* as a random effect. Note that these analyses only included individuals that survived until at least day 15, when infection status could be definitively determined and for whom we could measure a reproductive rate. w is the Akaike weight of each model.

Host-parasite combination	AICc	Δ AICc	w
<i>A. franciscana</i> infected with <i>E. artemiae</i>: time until sexual maturity			
Base model	692.3	0.0	0.58
Base model + Reproductive rate	694.0	1.6	0.26
Base model + Reproductive rate + Reproductive rate : Size class	694.9	2.6	0.16
<i>A. franciscana</i> infected with <i>E. artemiae</i>: probability of reproduction			
Base model + Reproductive rate	155.9	0.0	0.45
Base model	156.1	0.3	0.39
Base model + Reproductive rate + Reproductive rate : Size class	157.9	2.0	0.16
<i>A. franciscana</i> infected with <i>E. artemiae</i>: clutch type			
Base model	224.0	0.0	0.53
Base model + Reproductive rate + Reproductive rate : Size class	225.5	1.5	0.24
Base model + Reproductive rate	225.7	1.7	0.23
<i>A. franciscana</i> infected with <i>E. artemiae</i>: rate of offspring production*			
Base model + Reproductive rate + Reproductive rate : Size class	79.7	0.0	0.62
Base model + Reproductive rate	81.1	1.4	0.31
Base model	83.9	4.2	0.08
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>: clutch type			
Base model	110.6	0.0	0.74
Base model + Reproductive rate	112.9	2.4	0.22
Base model + Reproductive rate + Reproductive rate : Size class	116.5	6.0	0.04
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>: rate of offspring production*			
Base model + Reproductive rate + Reproductive rate : Size class	58.1	0.0	0.83
Base model	62.5	4.4	0.09
Base model + Reproductive rate	62.6	4.5	0.09

*Shown for the models that weighted nauplii and cysts equally; giving either offspring type a double weight produces qualitatively equivalent results.

Supplementary Table 8. Model comparison: link between survival and infection success. For each host-parasite combination, these models grouped all the individuals exposed to that parasite (possible outcomes: uninfected or infected) and the control individuals for that host into the factor *Resistant-Infected-Control* (abbreviated Res-Inf-Ctrl). *Resistant-Infected-Control* was allowed to interact with all of the experimentally manipulated factors (the base model). The base model for *A. franciscana* included *Sex***Size class* and a frailty component for *Origin* (lognormal distribution, see Results). The base model for *A. parthenogenetica* included *Size class* and a frailty component for *Batch* (log-logistic distribution, see Results). We used contrast manipulation to detect how resistant, infected and control individuals differed (only models within $\Delta AICc = 3$ of the best contrast-manipulated model are shown). Note that these analyses only included individuals that survived until at least day 15, when infection status could be definitively determined. *w* is the Akaike weight of each model.

Host-parasite combination	AICc	$\Delta AICc$	<i>w</i>
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>			
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Sex	1364.4	0	0.55
Contrast manipulation: Ctrl > Inf > Res	1364.4		
Base model + Res-Inf-Ctrl	1366.2	1.8	0.22
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Sex + Res-Inf-Ctrl : Size class	1366.7	2.3	0.17
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	1368.9	4.5	0.06
Base model	1427.6	63.2	0.00
<i>A. parthenogenetica</i> exposed to <i>E. artemiae</i> - low spore dose			
Base model + Res-Inf-Ctrl	641.5	0	0.65
Contrast manipulation: Inf > Ctrl = Res	640.6		
Inf > Ctrl > Res	641.5		
Inf = Ctrl > Res	642.3		
Base model	643.2	1.7	0.28
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	646.1	4.6	0.07

Supplementary Table 9. Overall tests: link between total number of offspring produced and infection success (factor *Resistant-Infected-Control*, abbreviated Res-Inf-Ctrl).

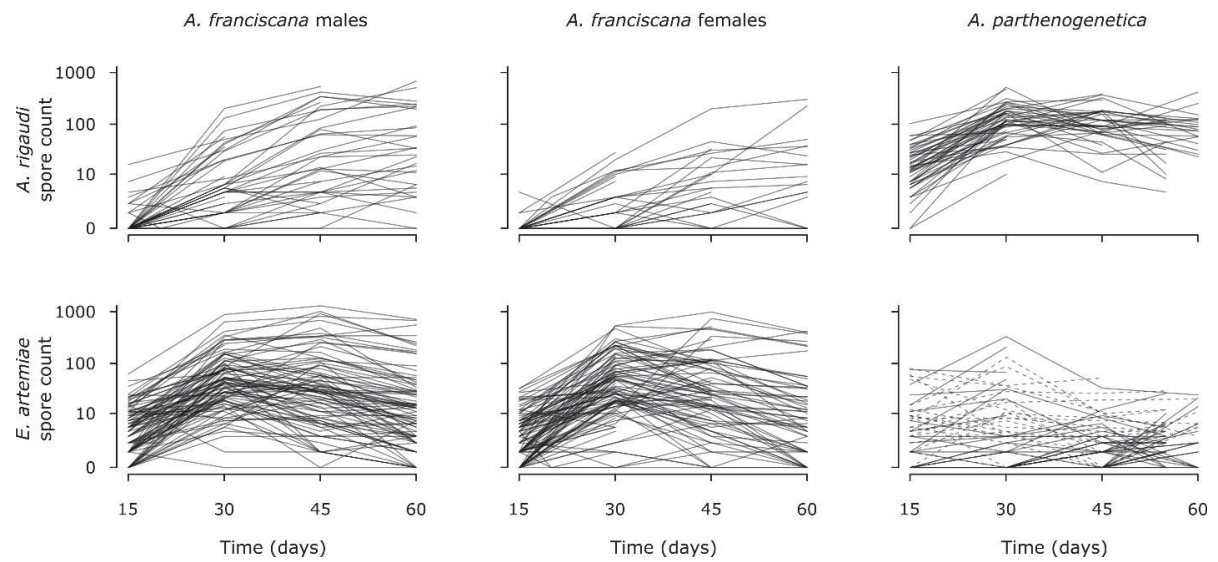
Host-parasite combination	df	$\chi^2(2)$	<i>p</i>
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>*			
Kruskal-Wallis test for overall difference between categories	2	48.1	< 0.0001
Dunn's post hoc test (Holm correction): Ctrl ≠ Inf			< 0.0001
Ctrl ≠ Res			< 0.001
Inf ≠ Res			0.73
<i>A. parthenogenetica</i> exposed to <i>E. artemiae</i>*			
Kruskal-Wallis test for overall difference between categories	3	3.2	0.28

*Shown for the tests that weighted nauplii and cysts equally; giving either offspring type a double weight produces qualitatively equivalent results.

Supplementary Table 10. Model comparison: link between reproduction and infection success. For each host-parasite combination, these models grouped all the individuals exposed to that parasite (possible outcomes: uninfected or infected) and the control individuals for that host into the factor *Resistant-Infected-Control* (abbreviated Res-Inf-Ctrl). *Resistant-Infected-Control* was allowed to interact with all of the experimentally manipulated factors (the base model). The base model for *A. franciscana* included *Size class* and *Origin* as a random or frailty component; the base model for *A. parthenogenetica* included *Size class* and *Batch* as a random effect. We used contrast manipulation to detect how resistant, infected and control individuals differed (only models within $\Delta AICc = 3$ of the best contrast-manipulated model are shown). Note that these analyses only included individuals that survived until at least day 15, when infection status could be definitively determined. *w* is the Akaike weight of each model.

Host-parasite combination	AICc	$\Delta AICc$	<i>w</i>
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>: time until sexual maturity			
Base model + Res-Inf-Ctrl	942.9	0	0.79
Contrast manipulation: <i>Inf = Res > Ctrl</i>	941.1		
<i>Inf > Res > Ctrl</i>	942.9		
<i>Inf > Res = Inf</i>	944.1		
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	945.2	2.6	0.21
Base model	966.4	23.5	0.00
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>: probability of reproduction			
Base model + Res-Inf-Ctrl	202.7	0	0.74
Contrast manipulation: <i>Ctrl > Inf = Res</i>	201.1		
<i>Ctrl > Inf > Res</i>	202.7		
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	204.8	2.1	0.26
Base model	238.4	35.7	0.00
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>: clutch type (higher = more nauplii)			
Base model + Res-Inf-Ctrl	303.8	0	1.00
Contrast manipulation: <i>Res > Inf > Ctrl</i>	303.8		
<i>Res = Inf > Ctrl</i>	304.5		
Base model	327.2	23.4	0.00
<i>A. franciscana</i> exposed to <i>A. rigaudi</i>: rate of offspring production*			
Base model + Res-Inf-Ctrl	95.7	0	0.68
Contrast manipulation: <i>Res > Ctrl = Inf</i>	95.3		
<i>Res > Ctrl > Inf</i>	95.7		
Base model	98.4	2.7	0.18
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	98.8	3.1	0.14
<i>A. parthenogenetica</i> exposed to <i>E. artemiae</i> - low spore dose: clutch type (higher = more nauplii)			
Base model + Res-Inf-Ctrl+ Res-Inf-Ctrl : Size class	320.7	0	0.49
Contrast manipulation: <i>order of effects dependent on Size class</i>	316.6		
<i>order of effects dependent on Size class</i>	318.5		
Base model + Res-Inf-Ctrl	321.1	0.4	0.40
Base model	323.8	3.1	0.10
<i>A. parthenogenetica</i> exposed to <i>E. artemiae</i> - low spore dose: rate of offspring production*			
Base model	181.9	0	0.94
Base model + Res-Inf-Ctrl	187.4	5.5	0.06
Base model + Res-Inf-Ctrl + Res-Inf-Ctrl : Size class	197.5	15.6	0.00

*Shown for the models that weighted nauplii and cysts equally; giving either offspring type a double weight produces qualitatively equivalent results.



Supplementary Figure 1. Spore production rates varied through time and across host-parasite combinations. Top: *A. rigaudi*, bottom: *E. artemiae*. The spore counts for each individual at day 15, 30, 45 and 60 (and some additional points at day 20 for *A. franciscana*) are connected. For *A. parthenogenetica* infected with *E. artemiae*, the low and high spore doses are represented by the solid and dashed lines, respectively. Spore count is on an $\ln + 1$ scale.

Supplementary methods

Statistical analyses: species-level effects

In general and unless otherwise specified, we included our experimentally manipulated factors in a full model, and used likelihood ratio tests to test their significance. If relevant, post-hoc comparisons for infection treatments were carried out using Dunnett's comparisons with a control (i.e. infected-with-*A. rigaudi* vs. controls, infected-with-*E. artemiae* vs. controls). To make inferences about effect sizes, we constructed predictive models containing only the significant factors. Analyses were run in R version 3.1.3 (R Core Team 2015) using the packages lme4 (linear mixed models, Bates et al. 2015), survival (survival analyses, Therneau 2014), and multcomp (function "glht" for post-hoc testing, Hothorn et al. 2008).

First, we analyzed the virulence of infections (effect on survival, growth, and reproduction). This was done separately for *A. franciscana* and *A. parthenogenetica*. The full models included the experimentally manipulated effects (*Treatment*, *Sex*, *Size class*) and the background grouping factors (*Origin* for *A. franciscana*; *Batch* for *A. parthenogenetica*). The spore dose had to be taken into account for *A. parthenogenetica* because we used two doses for *E. artemiae*; we treated these two as separate treatments. Importantly, we only analyzed virulence once we could be certain of individuals' infection status. To do this, we excluded all individuals that died before day 15 (see above), and only compared infected with control individuals. To make sure that we were not missing important events occurring before this cutoff, we repeated all statistical models for exposed vs. control individuals that died before day 15.

We analyzed host survival using parametric survival models. We established a full fixed-effects model for each host species, then determined the best-fitting parametric distribution (Weibull, exponential, extreme, Gaussian, logistic, lognormal, log-logistic, Rayleigh) using the corrected AIC. We then tested the significance of the predictive effects as described above. Finally, we confirmed the fit of the model by performing a goodness-of-fit test (comparing the likelihood of the observed data with the likelihood distribution of simulated datasets based on the model predictions). The full model for *A. franciscana* included *Treatment*, *Sex*, *Size class*, and all double interactions. The full model for *A. parthenogenetica* included *Treatment*, *Size class*, and their interaction. *Origin* and *Batch* were included as frailty components for *A. franciscana* and *A. parthenogenetica*, respectively, as they could introduce heterogeneity in mortality rates. Data were right-censored on day 60.

To test the effects of parasite infection on growth, we first checked whether there was significant growth between days 1 & 30 and days 30 & 60 (paired t-tests of the size difference between day 60 & 30 and day 30 & 1). Most growth occurred during the first month (see Results), so we only analyzed *Growth from day 1 to 30* further. For *A. franciscana*, we looked at the effects of the fixed effects *Sex*, *Treatment*, *Size class* and all their interactions, with *Origin* as a random effect. For *A. parthenogenetica*, the full model included *Treatment*, *Size class* and their interaction as fixed effects, and *Batch* as a random effect.

We analyzed (female) reproductive success in two steps. First, we did a simple overview analysis, comparing the total number of offspring produced by *A. franciscana* and *A. parthenogenetica* females in different *Treatments*. To circumvent skews in the data, we used non-parametric Kruskal-Wallis tests with Dunn's post hoc testing (R package PMCMR, Pohlert 2014). Offspring could be nauplii or cysts, and 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. Second, we decomposed female reproductive schedule into a) time until sexual maturity, b) the probability of producing a clutch, c) the type of offspring produced, and d) the rate and e) the timing of offspring production. The first two measures could not be tested for *A. parthenogenetica* females due to lack of variation. All models included *Treatment*, *Size class*, and their interaction as fixed effects, and *Origin* or *Batch* as random (or frailty) effects. The response variables were as follows. a) The number of days until females became sexually mature, with right-censoring in case of death. As above, we first determined the best parametric distributions to use, then tested the significance of the predictive effects. b) A binary variable describing whether a female produced a clutch during the experiment or not. c) For females that produced at least one clutch, a binomial combination of the number of clutches consisting of nauplii vs. cysts. d) For females that produced at least one clutch, the total number of offspring divided by the length of the reproductive period. The length of the reproductive period was defined as the difference between the date of death (or censoring) and the date of maturity. As above, we ran multiple models where nauplii were weighted twice, equally, and half as much as cysts, and based our conclusions on the overall pattern. e) This analysis examined the timing of females' investment into reproduction, for females that produced at least one clutch. Each clutch was a binomial data point (the size of the clutch relative to the total number of offspring produced by that female), modeled as a quadratic function of its clutch date. Clutch date was expressed as the elapsed proportion of the female's reproductive period (e.g. for two females reproducing on the 10th day of sexual maturity, where one died on the 20th day and one on the 40th, the elapsed proportions would be 0.5 and 0.25). *Individual* was included as a random variable to control for pseudoreplication.

Next, we analyzed the infections for parasite fitness components (spore production rate, transmission success, infectiousness of a single spore) and overall fitness. These analyses were combined for infections in *A. franciscana* and *A. parthenogenetica*.

We tested whether the rate of spore production varied through time, with parasite species, with host species, or depending on the host-parasite combination. We used *Spore count*, the number of spores counted in the fecal sample as the response variable in a generalized linear mixed model with a negative binomial distribution. We did not transform the spore count to spores/mL ($\approx \text{spore count} * 700$) to avoid skewing the error distribution. The fixed effects were *Day* (factorial), *Host species*, *Parasite species*, and all possible interactions; an individual-level

random effect was included to control for pseudoreplication. To avoid comparing apples with oranges, we excluded *A. parthenogenetica* that had been exposed to 10 000 *E. artemiae* spores from this model. However, we tested separately whether the rate of spore production differed for *A. parthenogenetica* infected with different doses of *E. artemiae* (equivalent model with fixed effects: *Day* (factorial), *Dose*, and the interaction). All spore production analyses were carried out for infected individuals only.

To investigate the relationship between spore production and transmission success, we compared the prevalence of infection in recipient individuals to the number of spores counted in the donor hosts' feces samples. We fit a generalized linear mixed model with a binomial response variable *Proportion of infected hosts* and fixed effects *Recipient species*, *Parasite species*, *Spore count*, and all possible interactions. We also included a random effect controlling for the pseudo-replication induced when one donor host was used multiple times (*Individual*). Originally, we included random effects to control for environmental variation (*Donor species* and *Day*), but these explained very little variance and caused the models not to converge, so they were removed. We could not test the effect of *Donor species* directly because the experiments for *A. franciscana* and *A. parthenogenetica* were not carried out at the same time.

We also used the transmission assay to estimate the infectiousness of a single spore in each host-parasite combination. To do so, we assumed that the establishment of microsporidian infections follows an independent-action model with birth-death processes. This model assumes that a parasite population grows in the host until it reaches an infective threshold, at which point the infection is considered to be established (Schmid-Hempel 2011 pp. 225–6). In our assay, we considered that an infection was established when we could detect it, i.e. the infectivity threshold corresponds to the threshold for PCR detection (estimated at ~1 000 spores inside the host's body, unpublished data). By explicitly accounting for within-host growth, the birth-death approach circumvents the problem of a short incubation time leading to false negatives for slow-growing infections. In these models, the probability per spore to start an infection, p , is equal to $-\ln\left(\frac{\text{noninfected}}{\text{exposed}}\right)/D$ where D is the spore dose (Schmid-Hempel 2011 pp. 225–6). In our transmission assay, D can be approximated by the number of spores in the fecal sample taken from the donor at the start of the assay (= spore count transformed to spores/mL, or * 700), divided by $5 \times 8 = 40$ (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, then calculated the average p (ln-transformed) for every host-parasite combination. We compared p across host-parasite combinations using non-parametric Kruskal-Wallis tests with Dunn's post hoc testing (R package PMCMR, ref).

Finally, we investigated parasite fitness in the different host-parasite combinations using two indicators. First, we summed the spores counts obtained on days 15, 30, 45 and 60 for each

infection; this produced a cumulative spore count. Second, we calculated an asymptotic growth rate for each infection 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 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 cumulative spore count is a measure of the basic reproduction number R_0 , which quantifies 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. We compared the two measures across host-parasite combinations using non-parametric Kruskal-Wallis tests with Dunn's post hoc testing (R package PMCMR, Pohlert 2014). *A. parthenogenetica* exposed to low and high spore doses of *E. artemiae* were treated separately.

CHAPTER 5

SPECIALIZED HOST EXPLOITATION CONSTRAINS THE EVOLUTION OF GENERALISM IN MICROSPORIDIAN PARASITES OF *ARTEMIA*

Eva J. P. Lievens, Yannis Michalakis[†] and Thomas Lenormand[†]

[†]equal contribution

Ecological specialization theory predicts that populations evolve towards specialism when environmental trade-offs are strong, and generalism when they are weak. Here, we used an experimental evolution approach to study the evolution of parasite specialization, and the underlying trade-offs, in two sympatric parasites: *Anostracospora rigaudi* and *Enterocytozpora artemiae*, microsporidians infecting the brine shrimp *Artemia franciscana* and *Artemia parthenogenetica*. We serially passaged the parasites on one, the other, or an alternation of the two hosts; after 10 passages, we assayed the infectivity, virulence, and spore production rate of the evolved lines. In accordance with previous studies, *A. rigaudi* maintained a higher fitness on *A. parthenogenetica*, and *E. artemiae* on *A. franciscana*, in all treatments. Our results suggest that infectivity in the two hosts is linked by a weak trade-off, allowing evolution towards generalism. However, this is counteracted by a strong trade-off in spore production across hosts, which dominates the final pattern of specialization. Therefore, the evolution of specialization in this system appears to be a product of several trait-specific trade-off shapes.

In progress.

INTRODUCTION

Ecological specialization is thought to be a major driver of biological diversity, and its evolution has been extensively studied by theoreticians and empiricists alike (reviewed in e.g. Futuyma and Moreno 1988, Kassen 2002, Ravigne et al. 2009, Lenormand 2012). A keystone of specialization theory is the assumption that adaptation to one environment comes at a cost of performance on other environments, creating fitness trade-offs (through antagonistic pleiotropy or the accumulation of deleterious alleles, Levins 1968, Kawecki 1994; reviewed in Kassen 2002, Hereford 2009). The strength of the fitness trade-offs determines, to a large degree, whether specialist or generalist strategies will evolve: strong trade-offs favor the evolution of specialists, while weak trade-offs favor the evolution of generalists with intermediate fitness (Van Tienderen 1991, Ravigne et al. 2009). The outcome of selection will depend further on the spatial and temporal homogeneity of the environment, with fluctuating environments in particular favoring the evolution of generalism (Bell and Rebourd 1997, Egas et al. 2004, Ravigne et al. 2009), as well as on the demography of the evolving system (Ronce and Kirkpatrick 2001, Ravigne et al. 2009).

Studies of ecological specialization have been especially fruitful in parasites and phytophagous insects. Multiple studies have revealed trade-offs in adaptation between hosts (e.g. Ebert 1998, Turner and Elena 2000, Via and Hawthorne 2002, Yourth and Schmid-Hempel 2006, Legros and Koella 2010; although see mixed support in e.g. Fry 1996, Agrawal 2000, Nidelet and Kaltz 2007, Magalhães et al. 2009, Bedhomme et al. 2012, Messina and Durham 2015), and others have demonstrated that generalist strategies are favored in fluctuating environments (e.g. Poullain et al. 2008, Bruns et al. 2014, Magalhães et al. 2014).

In this study, we used an experimental evolution approach to investigate the evolution of specialization, and the underlying costs of adaptation, for two sympatric parasites and their natural hosts. The microsporidians *Anostracospira rigaudi* and *Enterocytozpora artemiae* are ecologically similar parasites that infect the brine shrimp *Artemia parthenogenetica* and *Artemia franciscana* in the salterns of Southern France (Lievens et al. in prep.a, Rode et al. 2013a). The two parasites can complete their life cycles on both hosts, and commonly infect both hosts in the field (Lievens et al. in prep.a). Nonetheless, *A. rigaudi* and *E. artemiae* each show some degree of specialization, with *A. rigaudi* attaining a much higher fitness in *A. parthenogenetica*, and *E. artemiae* in *A. franciscana* (Lievens et al. in prep.b, in prep.a). We subjected *A. rigaudi* and *E. artemiae* to selection for generalism or specialism by serially passaging them on one, the other, or an alternation of the two hosts. We then assayed the infectivity, virulence, and spore production rate of the evolved lines, and asked the following questions: 1) did passaging treatment affect parasite fitness in each host species?; if so, 2) were there costs of adaptation?; and 3) were the changes in fitness caused by changes in infectivity, virulence, or the rate of spore production?

METHODS

Hosts and parasites

Natural system

Artemia is a genus of small crustaceans occurring in hypersaline environments. Our study system, the saltern of Aigues-Mortes on the Mediterranean coast of France, contains two coexisting *Artemia* species. The first, *A. parthenogenetica*, is an asexual clade native to the area; the second, *A. franciscana*, is a sexual species that was introduced from North America in 1970 and has since become highly prevalent (Amat et al. 2005, Rode et al. 2013c).

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

Origin of experimental parasites

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

The *A. rigaudi* and *E. artemiae* inocula used to start the serial passages were obtained by collecting the feces produced by the stock hosts over a 20-hour period. We collected the fecal solutions, homogenized them, and estimated their spore concentration as described by Lievens et al. (in prep.b).

Experimental evolution

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

Experimental conditions

We used lab-raised, parasite-free *Artemia* as experimental hosts for both the serial passages and the final assays. The experiment required a constant supply of fresh hosts; to meet this demand we collected live larvae from stock populations of Aigues-Mortes *A. franciscana* and *A. parthenogenetica* several times a week. Larvae were then raised separately until they could be used for the experiment. We controlled for host age and size as much as possible, by using 6 ± 1 -week old, (sub)adult *Artemia* for all passages and for the final assays. The only exceptions were passages P4, P5, and to some extent P8 (the latter for *A. franciscana* only), when episodes of lab-wide mortality decimated stock populations. In these cases, we used hosts of all ages. *A. franciscana* experimental hosts were always a random mix of males and females.

All *Artemia* were maintained at 23°C, in a parasite-free saline medium obtained by diluting autoclaved concentrated brine (Camargue Pêche, France) to 90 ppt with deionized water. *Artemia* were fed a solution of freeze-dried microalgae (*Tetraselmis chuii*, Fitoplankton marino, Spain; concentration: 6.8×10^9 cells/L deionized water). Stock hosts and hosts being raised for use in the experiment were fed *ad libitum*; assay hosts were fed 0.5 mL three times a week (~0.64 mL corresponds to the maximal ingestible amount for an adult *Artemia* in two days, Reeve 1963). Host groups in the serial passage experiment were fed at the same rate (20 mL per group per two days). For practical reasons, we did not adjust this quantity to host survival, so that hosts in high-mortality groups were probably slightly better fed than those in low-mortality groups. The serial passages and final assays took place under constant light.

Serial passages

We subjected *A. rigaudi* and *E. artemiae* to serial passaging under three evolutionary treatments: 'Single host *A. f.*', 'Single host *A. p.*', and 'Alternating hosts'. In the first two regimes, the parasites encountered only *A. franciscana* or only *A. parthenogenetica*; in the third regime, the parasites encountered alternating passages of *A. franciscana* and *A. parthenogenetica*. Each microsporidian \times treatment combination was replicated four times, producing a total of 24 parasite lines.

Parasites underwent ten serial passages, each lasting three weeks. To inoculate the first passage, P1, we used spore doses which were calculated to produce a maximal level of infection (as measured by Lievens et al. in prep.b): 3 000 spores/individual for *A. rigaudi* on both hosts and for *E. artemiae* on *A. franciscana*; 8 000 spores/individual for *E. artemiae* on *A. parthenogenetica* (10 000 spores/individual would have been more appropriate, but *E. artemiae* spores were limiting). For each parasite line, a group of 40 hosts was first exposed to spores in a limited volume of saline medium (300 mL). After two days, the volume was increased to 750 mL, and the infection was allowed to incubate for the rest of the passage time (19 days). After this incubation period, the spores produced by the surviving P1 hosts were used to infect the second passage, P2, as follows: for each parasite line, adult P1 hosts were placed in a strainer which allowed feces (and spores) to escape, but not the hosts themselves. This strainer was

suspended over the 40 new (P2) hosts in 1000 mL medium for 2 days. The strainer was then removed, the P1 hosts counted and stored in 96 % ethanol, and the P2 hosts placed in 750 mL fresh medium to incubate the infection for the rest of the passage time (19 days). Infections from P1 to P2 thus occurred directly from old to new hosts (a similar method was used by Rode et al. 2013a and Lievens et al. in prep.a). This method was repeated for P3 to P10. On occasion, a high proportion of the new hosts died during the two days of exposure to the old hosts. To prevent stochastic loss of parasite lines, if 5 or fewer new hosts survived, we added five additional new hosts to the group at the beginning of the incubation period. If all new hosts died, the transmission step was repeated for that line.

Two aspects of our passaging protocol should be pointed out: first, the time between passages (three weeks) is enough to allow infections to be transmitted between hosts (Rode et al. 2013a). Second, our method did not allow us to control for the number of spores that were transmitted from one group of hosts to the next. Thus, the size of the inoculum and of the microsporidian population in all passages after P1 were dependent on the infection dynamics that developed within each replicate line. At three points in the experiment, we estimated the within-host microsporidian population size at the time of transmission by counting the spore load in the surviving hosts. These counts were done for the P1, P4, and P7 hosts. After passaging (to P2, P4 and P8, respectively), half of each line's hosts were rinsed and ground up in 1 mL deionized water. We then added 429 μL of pure Ludox (Sigma-Aldrich, USA) and vortexed well, resulting in a homogeneous 30 % Ludox sample. Each sample was centrifuged for 15 min at 10 000 g, after which 1 179 μL of the supernate was removed. To rinse the sample, we added 750 μL deionized water, centrifuged for 4 min at 7 000 g, and removed 900 μL of the supernate. The remaining 100 μL contained the purified spores, which we stained with 1 μL 1X Calcofluor White Stain (18909 Sigma-Aldrich, USA); we then counted the number of spores in 0.1 μL using a fluorescence microscope (see Lievens et al. in prep.b for details). We estimated the microsporidian population size as the total number of spores in the body of all hosts = the spore count * 2 (because we only used half of the surviving hosts) * 1 000 (because we counted only 0.1 μL of the sample); this measure should reflect the size of the infective population (Refardt and Ebert 2006).

After the transmission step between the P6 and P7 hosts, we collected a backup spore sample for each parasite line. We kept the surviving P6 hosts from each line in 150 mL containing 10 mL algal solution for one week, after which we collected their feces and stored them at 4°C. Because storage at 4°C likely induces bottlenecks, this cannot be seen as an entirely representative snapshot of the microsporidian population at P6, but the alternative ('storage' in live hosts) would have caused further evolution in the samples. For the line *E. artemiae*/‘Single host A. f.’ – Replicate 4, feces were collected from the P5 hosts instead of the P6 hosts, because the P6 passage had to be skipped (see Results).

Final assays

At the end of the serial passage experiment, we tested the infectivity, virulence, and spore production of each evolved line in both *A. franciscana* and *A. parthenogenetica*. We tested all surviving parasite lines based on the spores they produced at the end of P10, and an additional subset of the parasite lines based on the spores we sampled from the P6 hosts (see Results).

To collect spores from the P10 hosts (after they had incubated the infection for three weeks), we placed each evolved line's hosts in a conical container containing 80 mL saline medium and 0.32 mL algal solution per individual (the maximum amount one adult *Artemia* can ingest over a 20 hour period, Reeve 1963), and allowed their feces to accumulate at the bottom of the container. After 20 hours, we removed the hosts and collected 4 mL of fecal solution. The fecal solution was homogenized, and a 100 μ L subsample was used to estimate the spore concentration (after dilution to 1 mL with 900 μ L deionized water). Homogenization and spore counting were carried out as described by Lievens et al. (in prep.b).

Spores collected from the P6 hosts were not used directly in the final assays because spore survival at 4°C is not indefinite (unpublished data). Instead, the spores were used to infect a set of 'revival' hosts 6 weeks after their collection, allowed to incubate for three weeks, and re-stored at 4°C. First, we quantified the spore concentration in each P6 sample by homogenizing, staining, and counting a 1 mL subsample as previously described for single samples (Lievens et al. in prep.b). We then infected a group of 20 hosts (*A. franciscana* for all revived lines except *E. artemiae*/‘Single host *A. p.*’ – Replicate 3) with 60 000 spores and allowed the infection to incubate for three weeks. Revival hosts were 5 ± 1 weeks old, and were kept in 250 mL of saline medium under the same conditions as experimental hosts. After three weeks, the revival hosts were placed in conical containers containing 100 mL saline medium, and feces were allowed to accumulate for one week. Accumulated feces were collected and stored at 4°C until the time of the final assays (10 days). At this time, we concentrated the fecal solutions to 4 mL by centrifuging them at 5 000 g for 8 min and removing the supernate; the 4 mL fecal solutions were then homogenized and the concentration measured as above.

A first, short-term assay tested the infectivity of each evolved line. For each line, we exposed 40 uninfected *A. parthenogenetica* and 40 uninfected *A. franciscana* to a low dose of the spores produced by the P10 or revival hosts. The assay hosts were placed in individual hemolymph tubes containing 1.5 mL saline solution and 750 spores; this dose was designed to produce a general level of 20-25% infection while being comparable across treatments (Lievens et al. in prep.b). After two days in this low volume, we could be sure that hosts had ingested all of the spores (Reeve 1963b), and a further 2.5 mL saline medium was added to the tube. The infection was allowed to incubate for five additional days, after which surviving individuals were sacrificed and tested for infection by PCR (Rode et al. 2013a). Seven days should not be enough time for differential mortality to affect the outcome of infectivity (Lievens et al. in prep.b).

In parallel with the first, a long-term assay tested the virulence and spore production of each evolved line. For each line, we exposed 40 uninfected *A. parthenogenetica* and 40 uninfected *A. franciscana* to a saturating spore dose, and tracked the mortality and spore production of these individuals for 90 days. The assay hosts were exposed in individual *Drosophila* tubes containing 1.5 mL saline solution and 3 000 spores; this dose was designed to produce a maximal level of infection while remaining comparable across treatments (Lievens et al. in prep.b). Because we suspected that this dose would lead to fewer infections when *A. parthenogenetica* was exposed to *E. artemiae*, we increased the level of replication for these combinations, exposing an additional 20 uninfected *A. parthenogenetica* to the parasite. After two days, 10 mL saline solution was added to the tube, and the infection was allowed to incubate until the host's death or until 90 days had passed. The saline medium was replaced once a week. Deaths were noted daily, and dead individuals were collected and stored in 96 % ethanol. Surviving individuals at the end of the assay were sacrificed and stored in 96 % ethanol. All individuals were then tested for infection by PCR following Rode et al. (2013a). Note that for dead individuals collected during the experiment, only positive PCR results are definitive because their DNA may degrade quickly.

In the long-term assay, transmission was measured by quantifying the number of spores produced by infected individuals over a two-day period after three weeks of infection¹; this corresponds to the window for transmission during the serial passage experiment. Two days after changing the saline medium on day 21, we collected 800 µL of each host's feces (containing spores) from the bottom of their tube. These samples were stored at 4°C until the infection status of each individual was known. Then, for each line, we pooled all fecal samples produced by infected *A. franciscana* and *A. parthenogenetica*. We quantified the spore count of 1 mL subsamples as described in Lievens et al. (in prep.b). By pooling samples, we minimized the error introduced by the sampling method, which is probably quite variable (since it depends on e.g. the consistency of the feces, the rate at which the host disturbs them, etc.).

Statistical analyses

All analyses were carried out in R version 3.1.3 (R Core Team 2015), using the packages lme4 (linear mixed modeling, Bates et al. 2015) and survival (survival analyses, Therneau 2014). Unless stated otherwise, we built full models with the relevant experimental factors, and tested for the significance of effects using the likelihood ratio test.

Serial passages

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

¹ Eventually, spore production will be quantified at more than one time point (the samples have been collected, but not analyzed). At the time of writing, however, this has not yet been done.

experiment.

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

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

Final assays

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

Infectivity was analyzed as the proportion of infected individuals at the end of the short-term assay (a binomial response). Both virulence and spore production were analyzed using only individuals who tested positive for infection. For virulence, we also excluded any hosts that died in the first 5 days of the experiment, because this is generally too early to detect infection (Rode et al. 2013a). Effects of *Treatment* on virulence were tested using Cox proportional hazard survival models, which fit the data well. To analyze the effects on transmission, we used the average spore production per infected host as a negative binomial response variable in a generalized linear mixed model.

Finally, we used spore production and infectivity to produce a composite fitness measure for each line. We used a measure of fitness that was representative for the context of the experiment, being the projected number of infections occurring if the line were passaged onto a new set of susceptible hosts. We calculated this as the total number of spores produced by the

surviving (infected) individuals over a two-day period after three weeks of incubation, multiplied by the infectiousness of a single spore. Infectiousness, the probability of a single spore to start an infection, was calculated based on the results of the short-term assay. Following an independent action model with birth-death processes, the 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 (Schmid-Hempel 2011 pp. 225–6). We analyzed fitness using a linear mixed model, after Box-Cox transformation ($\lambda = 0.21$ for *A. rigaudi*, $\lambda = 0.09$ for *E. artemiae*; values were too overdispersed to use Poisson regression, and there was not enough data to use negative binomial models).

RESULTS

Serial passages

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

Table 1. Evolved lines that were lost during the experiment. Underlined are the hosts from which we collected a backup spore sample. One of the lines, *A. rigaudi*/'Single host *A. f.*' – Replicate 2, could be revived from the backup spores and was used in the final assays.

Evolved line	Lost between	Details
<i>E. artemiae</i> /'Single host <i>A. p.</i> ' – Repl. 3	<u>P6</u> & P7	Spores collected from P6 hosts, but could not be revived
<i>A. rigaudi</i> /'Single host <i>A. f.</i> ' – Repl. 2	<u>P6</u> & P7	Spores collected from P6 hosts & revived
<i>A. rigaudi</i> /'Single host <i>A. f.</i> ' – Repl. 3	P4 & <u>P6</u>	No spores collected from P6 hosts
<i>A. rigaudi</i> /'Alternating hosts' – Repl. 4	P1 & P4	No spores collected from P6 hosts

At the beginning of passages P5, P6, and P7, exceptionally high mortality occurred in several groups of new hosts as they were being exposed to the parasites produced by the old hosts (Table 3). These episodes were concentrated in the treatments 'Single host *A. f.*'. Notably, the passaging step from P5 to P6 was repeated three times without success for the line *E. artemiae*/'Single host *A. f.*' – Replicate 4. The transfer was eventually achieved after 6 weeks of incubation in the P5 hosts, as the other lines were being passaged from P6 to P7. We denote this transfer as 'P5 → P7' for clarity, but P7 is only the 6th passage for this particular line. To investigate whether these effects were due to increased virulence or demographic effects (increased parasite load), we included backup spores produced by these lines in the final assays (see below).

Host survival was not constant throughout the serial passage experiment, even when the background variation in host quality was taken into account (Fig. 1). As the passages progressed, the survival of *A. franciscana* in 'Alternating hosts' lines increased as compared to 'Single host *A. f.*' lines; this effect was even more pronounced for *A. rigaudi* lines (significant

Table 2. Parasite lines that had exceptionally high mortality during one or more transmission events. Underlined are the hosts from which we collected a backup spore sample. All of the *E. artemiae*/‘Single host *A. f.*’ lines were revived from the backup spores and used in the final assays.

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

interaction between *Parasite species*, *Treatment*, and *Passage number*, $p < 0.01$, Supp. Table 1). Because we could not separate the background host mortality from parasite-induced effects, we cannot say whether this change was due to increasing parasite-induced mortality in the ‘Single host *A. f.*’ lines, or to decreasing parasite-induced mortality in the ‘Alternating hosts’ lines. For *A. parthenogenetica*, survival rates became progressively higher in *A. rigaudi* relative to *E. artemiae* lines, as well as in ‘Alternating hosts’ relative to ‘Single host *A. p.*’ lines (significant effects of *Parasite species* and *Treatment* in interaction with *Passage number*, $p < 0.001$ & $p < 0.01$, Supp. Table 1). Again, we could not distinguish between positive changes in *A. rigaudi* and ‘Alternating hosts’ lines or negative changes in *E. artemiae* and ‘Single host *A. p.*’ lines.

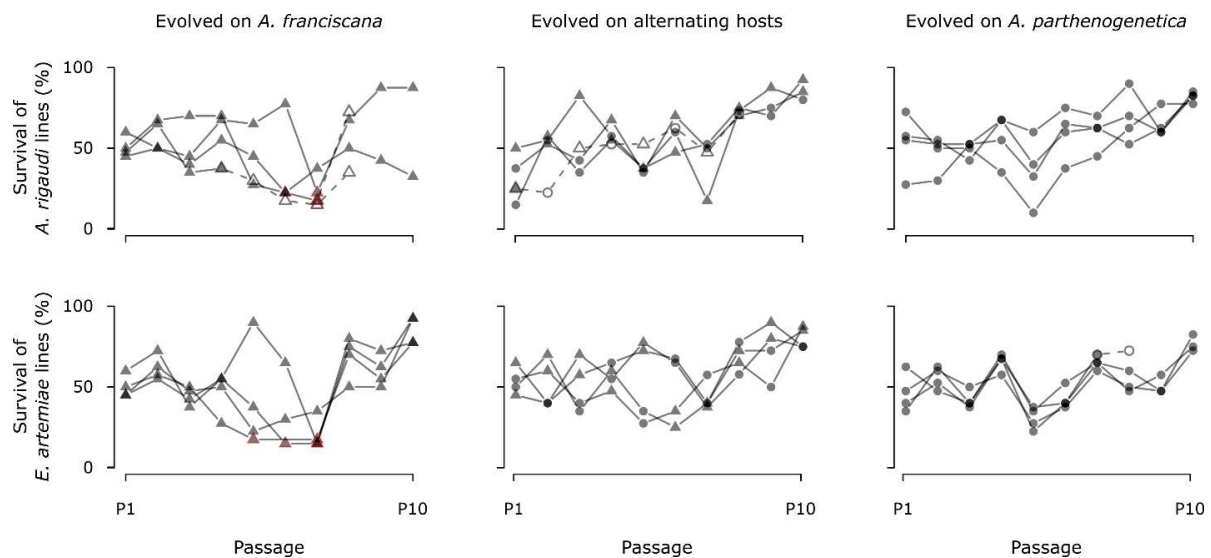


Figure 1. Host survival during the serial passage experiment (a compound of background host mortality and parasite-induced mortality). Top: *A. rigaudi* lines; bottom: *E. artemiae* lines. Triangles indicate *A. franciscana* hosts, circles indicate *A. parthenogenetica* hosts; points are connected per parasite line. Solid points and lines represent lines with ongoing infections, while empty points and dashed lines indicate lost lines (Table 1). Points are outlined in red if additional hosts were added after exposure, i.e. if survival is over-estimated (Table 3).

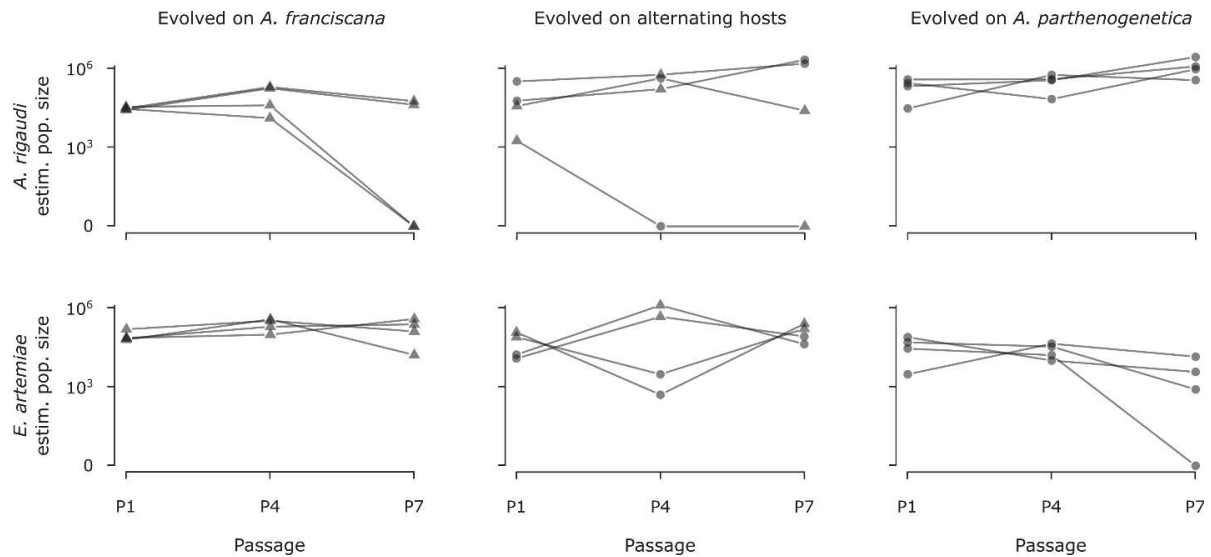


Figure 2. Parasite population size, as estimated for each passaging line. Top: *A. rigaudi*; bottom: *E. artemiae*; circles: *A. parthenogenetica* source host; triangles: *A. franciscana* source hosts. Lost lines (lines with an estimated population size of 0) were discarded from the analysis.

For *A. rigaudi*, the estimated within-host population size increased over the course of the experiment ($p < 0.01$ for *Passage number*; top row in Fig. 2), and was significantly higher for lines evolving on *A. parthenogenetica* than for lines evolving on *A. franciscana* ($p = 0.03$, Supp. Table 1). For *E. artemiae*, only the passaging regime impacted the population size, which was significantly higher in lines evolving on *A. franciscana* than in those evolving on *A. parthenogenetica* ($p < 0.01$, Supp. Table 1; bottom row in Fig. 2).

Final assays

During the final assays, we tested all surviving evolved lines, as well as a set of lines revived from the backup P6 spore samples (Table 3). These included all the lines in the parasite/treatment combination *E. artemiae*/‘Single host *A. f.*’, most of which experienced a period of exceptional mortality during the transmission events before the end of P6 (Table 3). The two *E. artemiae*/‘Alternating hosts’ lines whose P6 hosts were *A. franciscana* (Replicates 1 & 2) were also revived to act as controls for the effect of storage, but revival was only successful for Replicate 2. Finally, we succeeded in reviving the spores of the lost line *A. rigaudi*/‘Single host *A. f.*’ – Replicate 2.

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

Parasite species/Treatment	Replicate	Used?	Notes
<i>A. rigaudi</i> /'Single host <i>A. f.</i> '	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> /'Single host <i>A. p.</i> '	1	Y	
	2	Y	
	3	Y	
	4	Y	
<i>E. artemiae</i> /'Single host <i>A. f.</i> '	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 P6 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> /'Single host <i>A. p.</i> '	1	Y	
	2	Y	
	3	N	Lost.
	4	Y	

In the short-term assay, we tested for effects of passaging treatment on infectivity (Fig. 3, replicates shown in Supp. Fig. 1). The infectivity of *A. rigaudi* was unaffected by storage effects ($p = 0.21$, $\chi^2(1) = 1.6$), and did not change in response to passaging treatment ($p \geq 0.25$, Supp. Table 2); it tended to be higher in *A. parthenogenetica* ($p = 0.053$, Supp. Table 2). In contrast, the infectivity of *E. artemiae* was reduced by storage at 4°C ($p = 0.03$, $\chi^2(1) = 4.6$; dashed lines in Supp. Fig. 1), so the revived lines were excluded from further analysis. The infectivity of surviving *E. artemiae* lines was always higher in *A. franciscana* than in *A. parthenogenetica*, but the difference was less strong after passaging on 'Alternating hosts' and 'Single host *A. p.*' ($p = 0.02$ for interaction effect, Supp. Table 2).

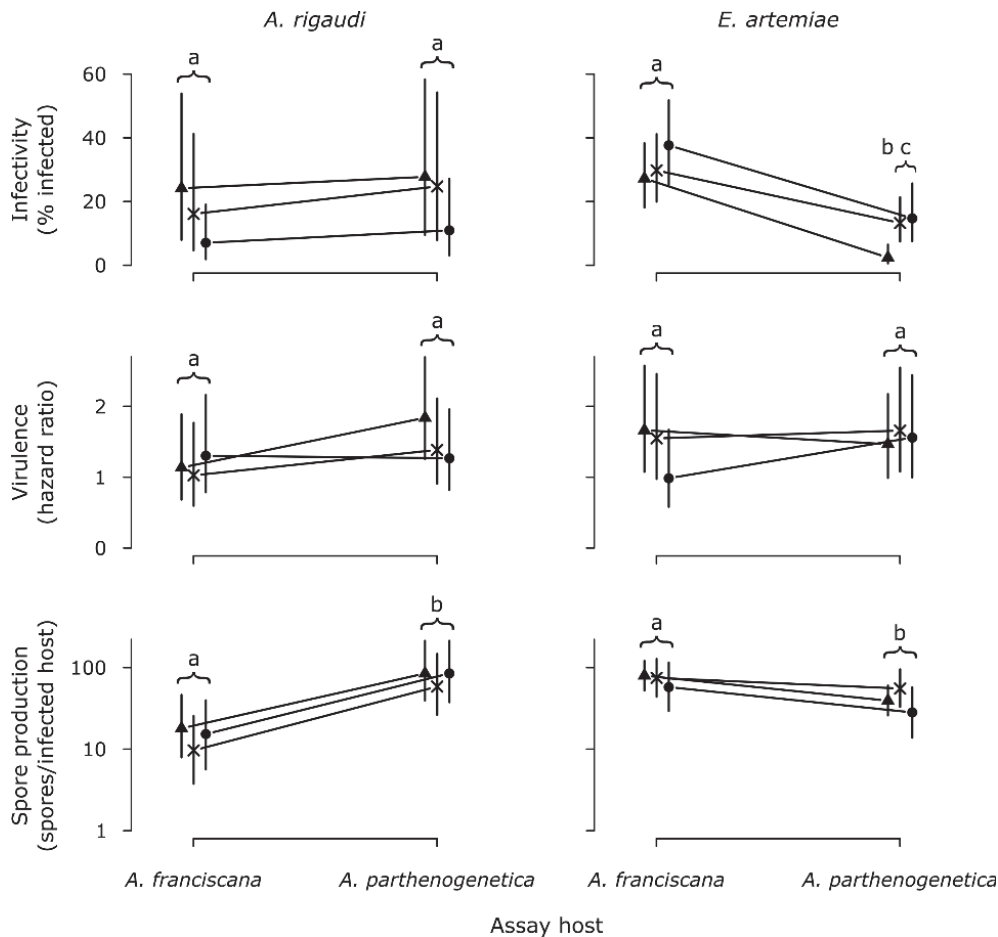


Figure 3. Infectivity, virulence, and spore production during the final assays. Infectivity is the percentage of hosts infected during the short-term assay. Virulence and spore production were measured in the long-term assay: virulence is the hazard ratio of each line compared to the unexposed controls of the same species, spore production is the number of spores produced per (surviving) infected host at the time of passaging (*ln* scale). Symbols denote the passaging treatment (triangles, 'Single host *A. f.*'; crosses, 'Alternating hosts'; circles, 'Single host *A. p.*'); vertical bars represent the 95% CIs. Significant differences are indicated by letters; analyses were done separately for each parasite.

In the long-term assay, we detected infection in a little over half of the exposed hosts in all host-parasite combinations except *A. parthenogenetica*-*E. artemiae* (Table 4). As expected, the detected prevalence in the long-term assay was a positive function of the infectivity in the short-term assay (Fig. 4), confirming the reliability of both results. The rate of detection was not significantly different for individuals that were collected after death (possibility of false negatives) than for sacrificed individuals (Table 4). Such a pattern is consistent with two underlying scenarios: a) the rate of infection did not differ between sacrificed and collected hosts, and the rate of false negatives in collected hosts was low; b) the rate of infection was higher in collected hosts, but compensated (nearly exactly) by a higher rate of false negatives. At this time, we cannot distinguish between these two scenarios.²

² Because we have kept individual fecal samples, we can confirm the status of collected hosts that tested negative for infection. At the time of writing, this has not yet been done.

Table 4. Detection of infection in the long-term assay, in hosts that were sacrificed at the end of the experiment or collected after their death during the experiment. Only hosts that died on or after day 6 are included here, to allow for the delay in detection time (Rode et al. 2013a).

Parasite species	Infection in sacrificed vs. collected hosts		$\chi^2(1)$	p
<i>A. rigaudi</i>				
<i>A. franciscana</i>	62 %	58 %	0.6	0.63
<i>A. parthenogenetica</i>	58 %	63 %	0.5	0.47
<i>E. artemiae</i>				
<i>A. franciscana</i>	60 %	55 %	1.1	0.29
<i>A. parthenogenetica</i>	21 %	27 %	3.6	0.06

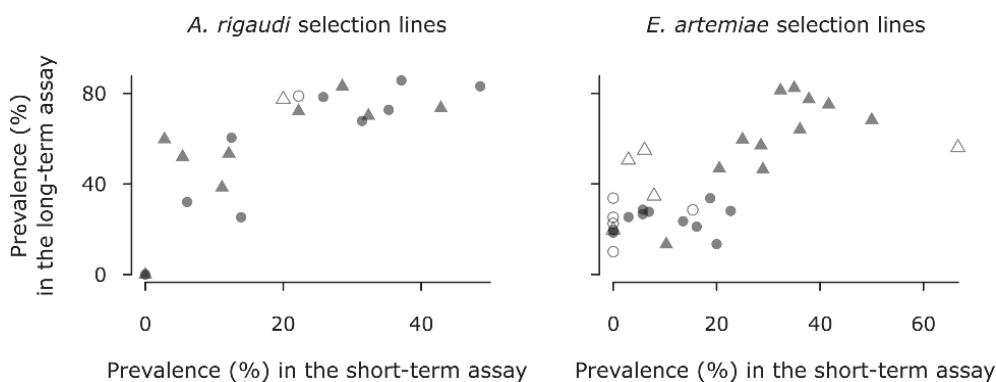


Figure 4. Correlation between prevalence of infection in the short-term and long-term assays. Prevalence in the long-term assay was subject to false negatives. Each evolved line is represented twice: once when assayed on *A. franciscana* (triangles), once when assayed on *A. parthenogenetica* (circles). Empty symbols represent revived lines. Only hosts that died on or after day 6 are included here, to allow for the delay in detection time (Rode et al. 2013a).

For both *A. rigaudi* and *E. artemiae*, parasite-induced mortality was unaffected by storage at 4°C ($p = 0.66$, $\chi^2(1.0) = 0.2$; $p = 0.11$, $\chi^2(3.2) = 6.2$, respectively). The revived lines were therefore included in the analyses. Mortality was always higher for *A. parthenogenetica*, but was not affected by passaging treatment, nor by the interaction between treatment and assay host ($p < 0.0001$, ≥ 0.23 , and $= 0.22$, respectively, Supp. Table 2; Fig. 3, replicates shown in Supp. Fig. 2). The line *A. rigaudi*/‘Single host *A. p.*’ – Replicate 1 could not be included in these analyses, because none of the hosts in the long-term assay were infected.

Similarly, spore production at passaging was unaffected by storage at 4°C ($p = 0.16$, $\chi^2(1) = 2.0$ for *A. rigaudi*; $p = 0.24$, $\chi^2(1) = 1.4$ for *E. artemiae*), so all lines were included in the further analyses. Spore production was higher in *A. parthenogenetica* for *A. rigaudi* and in *A. franciscana* for *E. artemiae* ($p < 0.01$ and < 0.01 , respectively, Supp. Table 2). However, there were no effects of treatment, nor of the interaction between treatment and assay host ($p \geq 0.55$ and ≥ 0.43 , respectively, Supp. Table 2; Fig. 3, replicates shown in Supp. Fig. 8). The line *A. rigaudi*/‘Single host *A. p.*’ – Replicate 1 could not be included in these analyses, because none of the hosts in the long-term assay were infected.

Finally, we analyzed an overall fitness measure for each line, quantified as the projected number of hosts that would be infected at passing (Fig. 5, replicates shown in Supp. Fig. 4). For *A. rigaudi*, there was no effect of storage at 4°C on fitness, so the single revived line was included in the analysis. We also included the line *A. rigaudi*/‘Single host *A. p.*’ – Replicate 1, which failed to infect hosts in the long-term assay, with fitness set to 0 (excluding the line did not change the results). *A. rigaudi* fitness was always higher when tested on *A. parthenogenetica*, with no effect of passing treatment, or of the interaction between treatment and assay host ($p < 0.01$, $= 0.29$, and $= 0.77$, respectively, Supp. Table 2). For *E. artemiae*, in contrast, the patterns of fitness mirrored those of infectivity. Fitness was lower after storage at 4°C ($p = 0.03$, $\chi^2(1) = 4.8$; dashed lines in Supp. Fig. 4), so the revived lines were excluded. *E. artemiae* fitness was always lower in *A. parthenogenetica*, but less so after passing on ‘Alternating hosts’ and ‘Single host *A. p.*’ ($p < 0.04$ for interaction effect, Supp. Table 2).

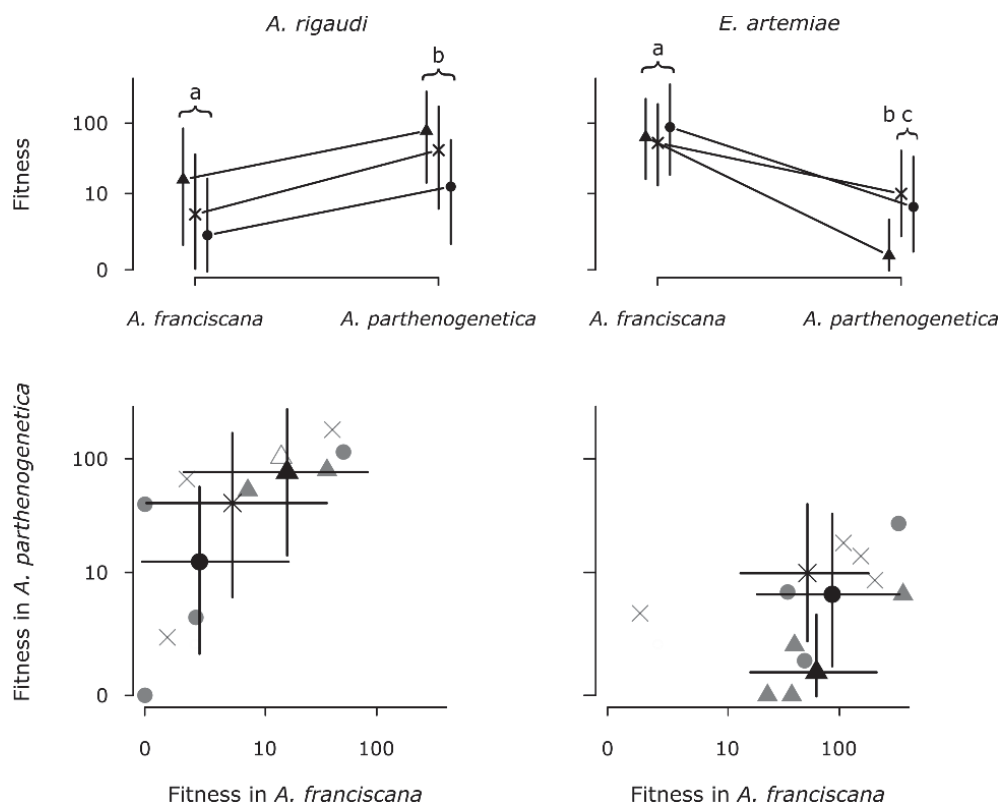


Figure 5. Fitness during the final assays (projected number of infections at passing, $\ln + 1$ scale), visualized in two classic ways (Bell and Reboud 1997). Top: fitness expressed as a reaction norm; bottom: performance in one host expressed as a function of performance in the other. Symbols denote the passing treatment (triangles, ‘Single host *A. f.*’; crosses, ‘Alternating hosts’; circles, ‘Single host *A. p.*’). Black points and vertical bars represent the mean and 95% CIs; gray points (lower graphs) show the values for each replicate (empty symbol: revived line). Significant differences are indicated by letters; analyses were done separately for each parasite.

DISCUSSION

We investigated the evolution of specialization, and the underlying costs of adaptation, in the microsporidian parasites *A. rigaudi* and *E. artemiae*. In the field, these parasites infect two species of *Artemia*, each with a degree of host specialization. *A. rigaudi* is preferentially adapted to *A. parthenogenetica*, and *E. artemiae* to *A. franciscana* (the “matched” hosts, Lievens et al. in prep.b), although all infection combinations are common in the field (Lievens et al. in prep.a). The parasites were passaged on one or both of their natural hosts over a period of ~7 months, after which we measured the infectivity, virulence, rate of spore production, and fitness of each evolved line.

The evolution of specialization and its underlying traits

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

A. rigaudi’s specialization for *A. parthenogenetica* was caused by a disparity in spore production. This parasite produced many more spores in *A. parthenogenetica* (by almost an order of magnitude, Fig. 3), but its virulence and infectivity did not differ between hosts. These results correspond well with previous findings: the overall specialization of *A. rigaudi* for *A. parthenogenetica* is driven by a specialization in spore production, not by any difference in infectivity (Lievens et al. in prep.b). None of *A. rigaudi*’s (measured) traits changed detectably during the serial passages, so the parasite stayed equally specialized for *A. parthenogenetica* in all treatments (Fig. 5).

For *E. artemiae*, specialization was apparent for spore production and infectivity. *E. artemiae* spores had a higher chance of infecting *A. franciscana*, and *E. artemiae* infections also produced more spores in *A. franciscana* (Fig. 3). Compounded, these two traits produce a clear pattern of specialization (Fig. 5). Unlike that of *A. rigaudi*, however, *E. artemiae*’s fitness did evolve in some treatments. *E. artemiae* lines whose passaging history included *A. parthenogenetica* had a higher fitness on this host, while their fitness in *A. franciscana* was unchanged (compare cross & circle to triangle in Fig. 5). *E. artemiae* can thus evolve cost-free generalism, supporting the mounting evidence that costs of adaptation are not universal (reviewed in Kassen 2002, more recently e.g. Nidelet and Kaltz 2007, Bedhomme et al. 2012, Remold 2012, Gallet et al. 2014), in particular when adapting to a poor environment (shown for e.g. spider mites, Agrawal 2000, Magalhães et al. 2009; seed beetles, Messina and Durham 2015; see also Falconer 1990). *E. artemiae*’s fitness change was driven by a change in infectivity, while virulence and spore production were static. Interestingly, changes in infectivity have also been found to drive the

evolution of specialists and generalists in the microsporidian *Brachiola algerae*, although in this case there was a correlated loss of infectivity in other hosts (Legros and Koella 2010).

The difference in infectivity among the evolved lines of *E. artemiae* can be interpreted in two ways: its infectivity in *A. parthenogenetica* either decreased when the parasite was no longer exposed to this host, or increased when the parasite was forced to persist in it. We consider the second to be more likely. The first possibility could be achieved by the accumulation of conditionally deleterious alleles (neutral in *A. franciscana*, deleterious in *A. parthenogenetica*; Kawecki 1994) or through a loss of genetic diversity (Yourth and Schmid-Hempel 2006). However, given the size of the initial inoculum and the limited timeframe of the experiment, we doubt that such processes occurred. The second hypothesis requires the increase in frequency of conditionally beneficial alleles (neutral in *A. franciscana*, beneficial in *A. parthenogenetica*), a process which is easier to achieve. In addition, it is supported by previous experimental results, which describe the infectivity of the stock population of *E. artemiae* as resembling that of the ‘Single host *A. f.*’ evolved lines (Lievens et al. in prep.b).

E. artemiae’s virulence did not differ among treatments at the end of the serial passaging, and we found no evidence that it evolved over the course of the experiment. In particular, we found no evidence that the high death rates caused by *E. artemiae* in *A. franciscana* between P4 and P6 were caused by a higher virulence (Fig. 1). Instead, demographic effects were the likely culprit: *A. franciscana* does not limit *E. artemiae*’s peak spore production to a certain maximum (Lievens et al. in prep.b), so a higher infective dose in this combination might lead to a higher peak transmission rate, which would increase the infective dose and so on, until the number of invading spores was so high that recipient hosts were overwhelmed (e.g. Ebert et al. 2000).

Do trait-specific trade-offs shape the specialization of A. rigaudi and E. artemiae?

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

The observed changes in infectivity can be explained by the existence of a weak trade-off between infectivity in *A. franciscana* and *A. parthenogenetica*. Consider first *E. artemiae*, whose ability to infect *A. parthenogenetica* improved when passaged on that host, without attendant losses in *A. franciscana*. Such cost-free adaptation could arise if the ancestral ‘*A. franciscana*-adapted’ infectivity of *E. artemiae* was located slightly below the boundary of a weak trade-off curve (such as would be expected if the ancestral population was not perfectly adapted to the conditions of the experiment, Fry 2003). There would then be little improvement possible in *E.*

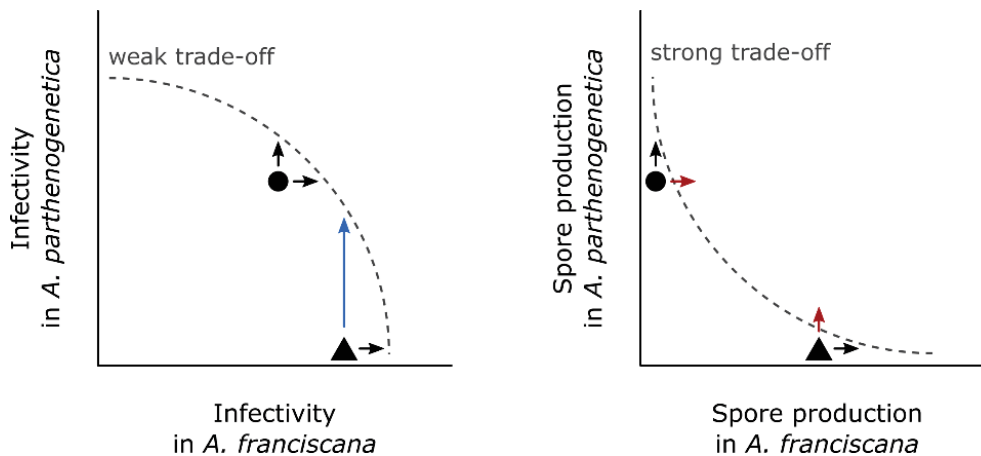


Figure 6. We speculate that the observed patterns of specialization in infectivity and spore production are determined by weak and strong trade-offs in performance between *A. franciscana* and *A. parthenogenetica* (see text for more information). Symbols: *A. rigaudi*, circle; *E. artemiae*, triangle.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

artemiae's fitness on *A. franciscana*, but a substantial improvement in *A. parthenogenetica* could easily be achieved (blue arrow in Fig. 6) (Martin and Lenormand 2015), as seen when *E. artemiae* was passaged on this host (Fig. 3). The infectivity of *A. rigaudi* can be interpreted in the same context. *A. rigaudi*'s ancestral infectivity is largely generalist (Lievens et al. in prep.b), so that the potential improvements in fitness would be small in either direction, thus producing the unchanged infectivity that we observed across treatments (Fig. 3).

The weak trade-off model implies that the evolution of generalist infectivity should be straightforward, begging the question of why *E. artemiae*'s ancestral population remained specialized for this trait. We speculate that the specialization of the ancestral *E. artemiae* population was maintained by source-sink dynamics in the natural host-parasite community. In the field, *E. artemiae* is present year-round, while *A. parthenogenetica* are only present from late spring to fall, so its population predominantly infects, and evolves on, *A. franciscana* (Lievens et al. in prep.a). In this case, adaptations towards increased infectivity in the mismatched host may be continually eroded by selection in the matched host (Lenormand 2002, Magalhães et al. 2007). By forcing *E. artemiae* to evolve on *A. parthenogenetica*, we blocked these source-sink dynamics, allowing generalist infectivity to evolve. In comparison, *A. rigaudi* almost exclusively occur in communities containing both host species (Lievens et al. in prep.a), potentially explaining why this microsporidian had already evolved generalist infectivity.

The second important trait for *A. rigaudi* and *E. artemiae* was spore production, which remained strongly specialized in all treatments (Fig. 3). This result could be explained by the existence of a strong trade-off in spore production between the hosts. Such a trade-off would allow small improvements in the direction of increased specialization (black arrows in Fig. 6),

but make improvements on the novel host much more difficult to achieve (red arrows in Fig. 6); it would also prevent the emergence of a generalist phenotype (Van Tienderen 1991, Ravigne et al. 2009). Mechanistically, such a strong trade-off could be related to the distinct strategies of host exploitation necessary to thrive in *A. franciscana* and *A. parthenogenetica*. The precise physiology of the host species is likely to be different (they have been diverging for an estimated 40 million years, Baxevanis et al. 2006), and indeed the mechanisms of virulence and within-host regulation employed by *A. rigaudi* and *E. artemiae* in their matched hosts differ (Lievens et al. in prep.b), with *A. rigaudi* causing more survival virulence, and *E. artemiae* more reproductive virulence. Successful exploitation of *A. franciscana* and *A. parthenogenetica* could therefore require very different toolkits, preventing the evolution of generalism and reducing the likelihood of a host switch (cf. Gemmill et al. 2000).

CONCLUSION AND PERSPECTIVES

In conclusion, we have found that the microsporidians *A. rigaudi* and *E. artemiae* are robustly specialized for respectively *A. parthenogenetica* and *A. franciscana*, and that this specialization is probably caused by a strong trade-off in spore production. Our results also indicate that infectivity, while playing a role in the degree of observed specialization, can readily evolve towards generalism – and indeed, has already done so in *A. rigaudi*. Interestingly, our conclusions are somewhat different from previous interpretations of the available field and experimental data, where we speculated that source-sink dynamics, coupled with a cost of generalism, were preventing *A. rigaudi* and *E. artemiae* from fully incorporating the mismatched hosts into their niche (Lievens et al. in prep.b, in prep.a). By studying the evolution of individual traits in a context of experimental evolution, we have instead found that *A. rigaudi* and *E. artemiae*'s continuing specialization is more likely to be caused by a strong trade-off in one particular trait (host exploitation). These results point to the idea that more theoretical studies of specialization should be set in a multi-trait context, with each trait able to exhibit weak or strong trade-offs and evolve accordingly. Such studies would be better equipped to describe the continuum between generalist and specialist strategies, and to single out the traits favoring their evolution.

NOTA BENE

This experiment is a work in progress, with two major elements of data collection remaining: 1) For those hosts that died during the long-term assay and tested negative for infection, we will distinguish true from false negatives by looking at individual fecal samples. If the rate of false negatives was dependent on treatment, this may affect the results. 2) Because transmission of *A. rigaudi* and *E. artemiae* varies through time, the rate of spore production at the time of passaging only gives us a piece of the puzzle. Some lines, for example, may have

evolved to produce a high spore count at precisely this time, while others evolved to produce spores early on. Therefore, we will quantify spore production at various points of the infection, and use these to calculate a rate of parasite growth (which can be used as a proxy for host exploitation), a peak spore count, and a peak spore time (as in Lievens et al. in prep.b). This will also provide us with better insights into any epidemiological feedbacks that may have occurred during the serial passaging.

ACKNOWLEDGEMENTS

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

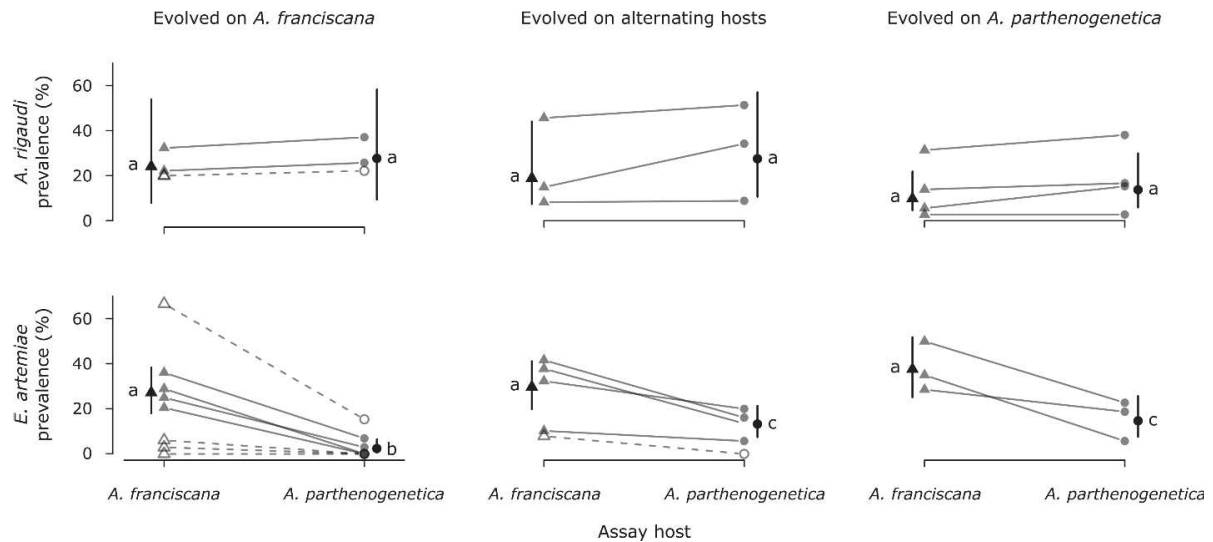
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Significance of the experimental variables for host mortality and parasite population size during the serial passages. *Treatment* refers to the passaging treatments ‘Single host *A. f.*’, ‘Single host *A. p.*’ and ‘Alternating hosts’. All models included parasite line as a random effect; host mortality models also contained *Passage* as a random factor. Significance was tested using the likelihood ratio test; if there were significant interactions, the independent contributions of the effects were tested separately using Type III sum of squares (Wald χ^2 test; in italics).

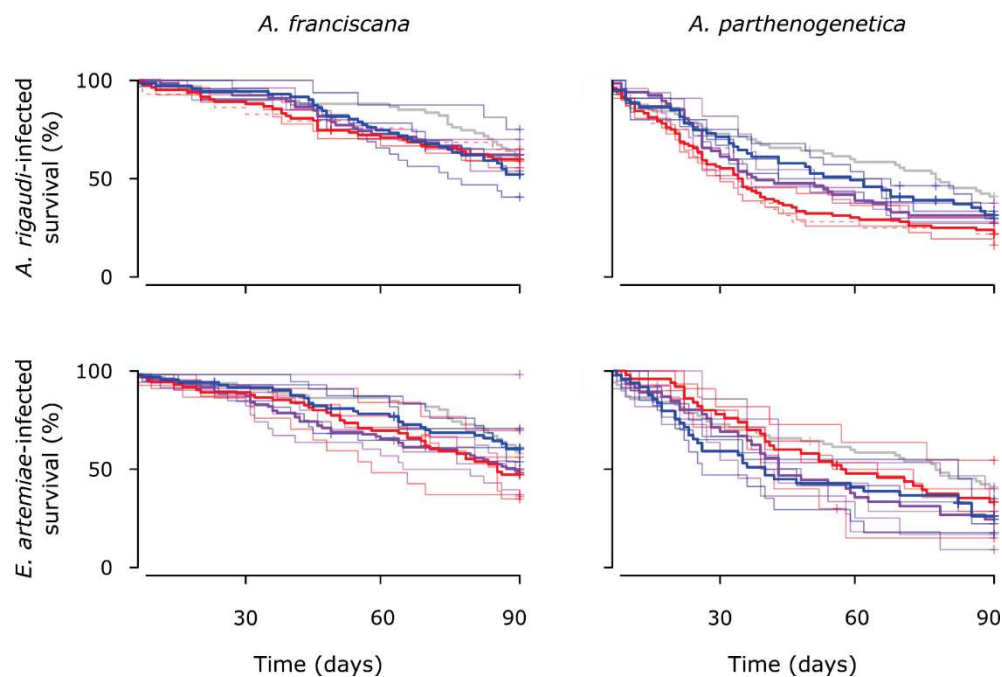
Response variable	Fixed effect	df	χ^2	<i>p</i>
Changes in host mortality				
<i>A. franciscana</i>	Treatment	1	2.8	0.09
	Parasite species	1	1.9	0.17
	Passage number	1	0.0	0.86
	Treatment : Parasite species	1	3.2	0.07
	Passage number : Treatment	1	20.9	< 0.0001
	Passage number : Parasite species	1	12.5	< 0.001
	Passage number : Treatment : Parasite species	2	7.0	< 0.01
<i>A. parthenogenetica</i>	Treatment	1	6.6	0.01
	Parasite species	1	3.1	0.08
	Passage number	1	22.7	< 0.0001
	Treatment : Parasite species	1	1.3	0.23
	Passage number : Treatment	1	8.6	< 0.01
	Passage number : Parasite species	1	11.4	< 0.001
	Passage number : Treatment : Parasite species	2	0.4	0.54
Changes in parasite population size				
<i>A. rigaudi</i>	Treatment	2	7.3	0.02
	Passage number	1	9.5	< 0.01
	Passage number : Treatment	2	2.3	0.32
<i>E. artemiae</i>	Treatment	2	10.5	< 0.01
	Passage number	1	0.0	0.93
	Passage number : Treatment	2	3.8	0.15

Supplementary Table 2. Significance of the experimental variables for fitness, infectivity, virulence, and spore production during the final assay. *Treatment* refers to the passaging treatments ‘Single host *A. f.*’, ‘Single host *A. p.*’ and ‘Alternating hosts’. All models included evolved line as a random or frailty effect. Significance was tested using the likelihood ratio test; if there were significant interactions, the independent contributions of the effects were tested separately using Type III sum of squares (Wald χ^2 test; in italics).

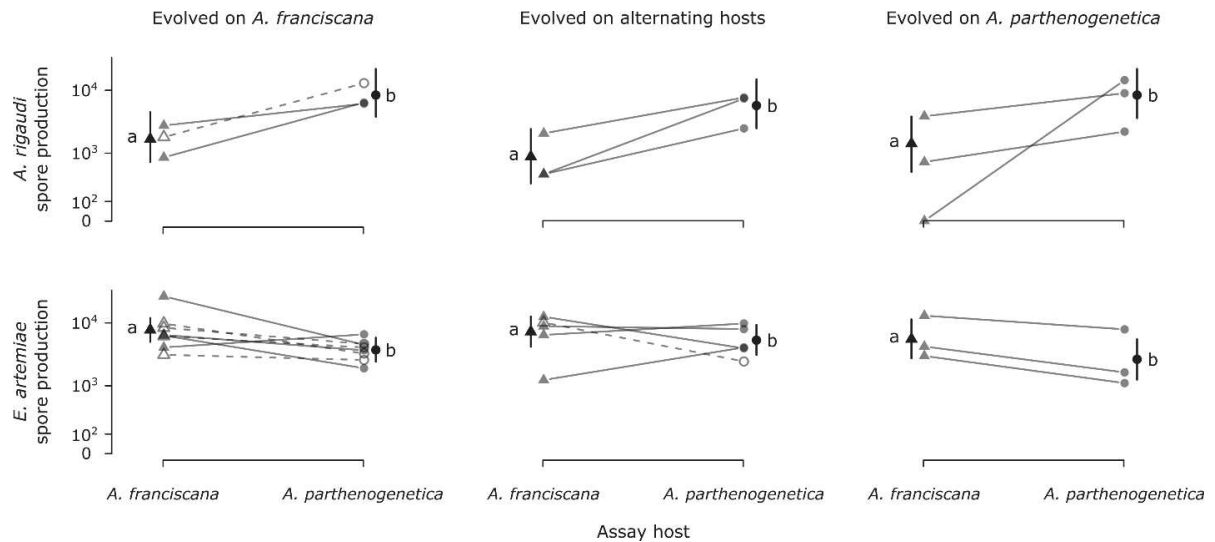
Response variable	Fixed effect	df	χ^2	<i>p</i>
Infectivity				
<i>A. rigaudi</i>	Treatment	2	2.8	0.25
	Assay host	1	3.7	0.05
(revived line included)	Treatment : Assay host	2	0.6	0.73
<i>E. artemiae</i>	Treatment	2	1.8	0.41
	Assay host	1	19.7	< 0.0001
(revived lines excluded)	Treatment : Assay host	2	8.1	0.02
Virulence				
<i>A. rigaudi</i>	Treatment	2.0	2.9	0.23
	Assay host	1	59.4	< 0.0001
(revived line included)	Treatment : Assay host	2	3.0	0.22
<i>E. artemiae</i>	Treatment	0.5	0.5	0.25
	Assay host	5.4	62.6	< 0.0001
(revived lines included)	Treatment : Assay host	1.9	2.9	0.22
Spore production				
<i>A. rigaudi</i>	Treatment	2	1.4	0.50
	Assay host	1	14.2	< 0.001
(revived line included)	Treatment : Assay host	2	0.1	0.96
<i>E. artemiae</i>	Treatment	2	1.7	0.43
	Assay host	1	9.1	< 0.01
(revived lines included)	Treatment : Assay host	2	1.2	0.55
Fitness				
<i>A. rigaudi</i>	Treatment	2	2.5	0.29
	Assay host	1	10.6	< 0.01
(revived line included)	Treatment : Assay host	2	0.5	0.77
<i>E. artemiae</i>	Treatment	2	0.2	0.90
	Assay host	1	34.9	< 0.0001
(revived lines excluded)	Treatment : Assay host	2	6.3	0.04



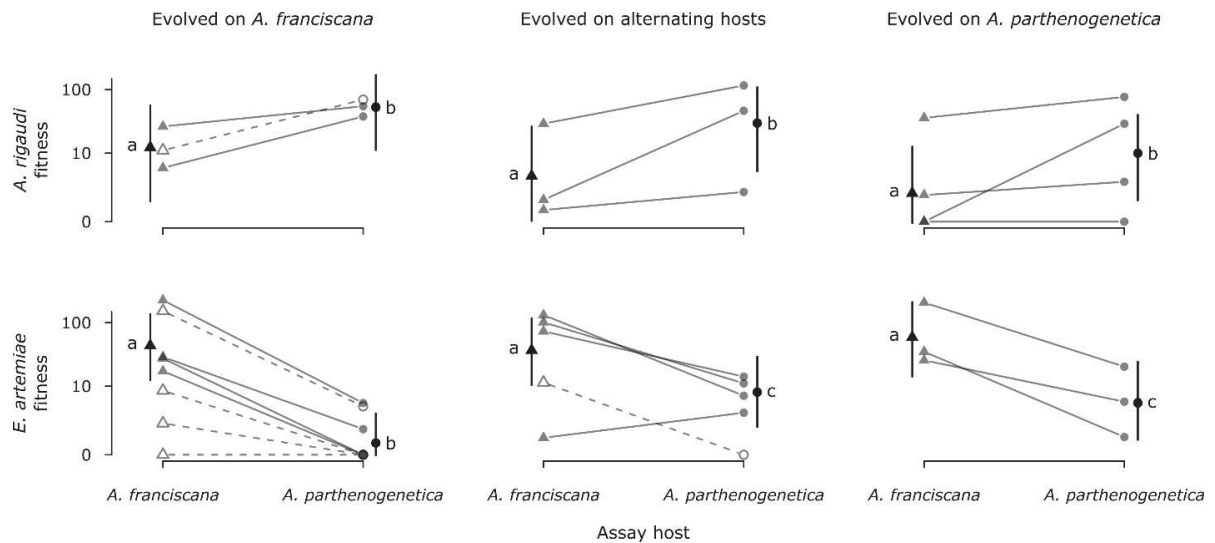
Supplementary Figure 1. Infectivity of the evolved lines during the final assays. Top: *A. rigaudi* lines; bottom: *E. artemiae* lines. Triangles indicate *A. franciscana* hosts, circles indicate *A. parthenogenetica* hosts; points are connected per parasite line. Solid points and lines represent surviving lines (at P10), while hollow points and dashed lines indicate evolved lines (P6 backup spores used). Mean infectivity is shown in black, with the vertical bar indicating the 95% CI. Significant differences are indicated by letters; analyses were done separately for each parasite and did not included the revived *E. artemiae* lines.



Supplementary Figure 2. Survival of infected hosts during the final assays (from day 6 onwards). Top: *A. rigaudi* lines; bottom: *E. artemiae* lines. Colors indicate *Treatment*: red, 'Single host *A. p.*'; purple, 'Alternating hosts'; blue, 'Single host *A. f.*'; gray, non-exposed controls. Translucent lines track the survival of individual infection lines; solid lines represent the average survival by *Treatment*. Dashed lines indicate revived lines (P6 backup spores used; only shown for *A. rigaudi*).



Supplementary Figure 3. Spore production during the final assays. Shown is the number of spores produced per (surviving) infected host after three weeks of incubation, over a two-day period. Top: *A. rigaudi* lines; bottom: *E. artemiae* lines. Triangles indicate *A. franciscana* hosts, circles indicate *A. parthenogenetica* hosts; points are connected per parasite line. Solid points and lines represent surviving lines (at P10), while hollow points and dashed lines indicate revived lines (P6 backup spores used). Mean spore production is shown in black, with the vertical bar indicating the 95% CI. Significant differences are indicated by letters; analyses were done separately for each parasite.



Supplementary Figure 4. Fitness (projected number of infections at passaging, $\ln + 1$ scale) of the evolved lines during the final assays. Top: *A. rigaudi* lines; bottom: *E. artemiae* lines. Triangles indicate *A. franciscana* hosts, circles indicate *A. parthenogenetica* hosts; points are connected per parasite line. Solid points and lines represent surviving lines (at P10), while hollow points and dashed lines indicate revived lines (P6 backup spores used). Mean fitness is shown in black, with the vertical bar indicating the 95% CI. Significant differences are indicated by letters; analyses were done separately for each parasite and did not included the revived *E. artemiae* lines.

Supplementary methods

Origin of parasites

We used the field-based laboratory stocks of *A. rigaudi* and *E. artemiae* used by Lievens et al. (in prep.b), who describe them as follows:

“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 maintain the infection. We selected both infected *A. franciscana* and infected *A. parthenogenetica* from the field, and maintained each stock population on a mix of *A. franciscana* and *A. parthenogenetica* hosts (n_{hosts} at any given time = ~20~50 per microsporidian species). Thus, our stocks contained a mix of spores from different field sites and times, collected from and propagated on both host species. Note that while we tried to maximize microsporidian diversity through this wide sampling, the genetic diversity of the stocks is unknown.”

Our field-based laboratory stocks of *A. rigaudi* and *E. artemiae* were susceptible to contamination with respectively *E. artemiae* and *A. rigaudi*, because we could not eliminate the possibility that a coinfecting host had been added accidentally. To ensure we were using an inoculum containing only *A. rigaudi* or *E. artemiae*, we purified our stocks in three steps:

- 1) We infected lab-bred *Artemia* with spore samples taken from the field-based stock populations at different times. For *A. rigaudi*, we used spores sampled from the stock population on 28/11/2014, 5/12/2014 and 13/2/2015. For *E. artemiae*, we used spores sampled from the stock population on 13/2/2015 and 25/2/2015. Each of these samples was taken by collecting the feces produced by the stock hosts during one week, and had been stored at 4°C since. For each of the five spore solutions, we infected 16 large, adult *A. parthenogenetica* and 32 medium-sized, juvenile *A. franciscana*. *A. parthenogenetica* were isolated and exposed to spores in 5 mL of saline medium for two days, the volume was then increased to 20 mL and the infection allowed to incubate for 12 extra days. *A. franciscana* were separated into pairs and exposed to spores following the same schedule as *A. parthenogenetica*. The experimental conditions were as follows: the saline medium was sterilized brine diluted to 90 ppt with deionized water, the temperature was 23°C, and feeding was *ad libitum*. For *E. artemiae*, we also used 22 *Artemia* sampled from the field on 12/3/2015. These individuals were used directly for step (2).

- 2) We selected spores produced by singly-infected *Artemia* only. After step (1), we ground up each individual *A. parthenogenetica* and each pair of *A. franciscana* in 100 µl deionized water. One quarter of each homogenate was tested for the presence of *A. rigaudi* and *E. artemiae* by PCR (following Rode et al. 2013a). Uninfected or coinfecting samples were discarded, while the remainders of the singly-infected homogenates were combined into one *A. rigaudi* sample and one *E. artemiae* sample. These samples were purified on a Ludox density gradient to remove debris as follows. Samples were divided into 2 mL batches, which were layered onto 1.5 mL of a 30 % Ludox (Sigma-Aldrich, USA) solution in 15 mL falcon tubes and centrifuged for 20 min at 7 000 g. We then replaced 3 mL of the supernate with fresh deionized water, vortexed the sample, and re-centrifuged for 4 min at 5 000 g. Finally, we removed 3 mL of the supernate, resulting in a purified and rinsed spore sample.
- 3) We used the recombined and purified homogenates described in step (2) to infect new populations of lab-bred and parasite-free *Artemia*; these new stock populations thus contained only *A. rigaudi* or only *E. artemiae*. For each microsporidian, the new, 'purified', stock population consisted of ~150 individuals, of which half were *A. parthenogenetica* and half were *A. franciscana*. These hosts were exposed to the spores together in 150 mL of sterilized brine for two days, then the hosts were transferred to the separating funnels and volume was increased to ~2.5 L. The purified stock populations were maintained in sterilized brine diluted to 90 ppt with deionized water, at 23°C and with *ad libitum* feeding.

Whereas the experiment described by Lievens et al. (in prep.b) used spores from both the field-based and the purified stock populations, this experiment only used spores produced by the latter.

DISCUSSION & PERSPECTIVES

The studies in this section offer a remarkably consistent view of the state, causes, and consequences of specialization in the microsporidians *A. rigaudi* and *E. artemiae*. The microsporidians can complete their life cycle in either *Artemia* host, but are otherwise rather specialized, having substantially higher fitness on their ‘matched’ hosts: *A. parthenogenetica* for *A. rigaudi*, and *A. franciscana* for *E. artemiae* (Chapter 4). This trait is evolutionarily robust, as both microsporidians retained their specialization after seven months of exclusive evolution on the ‘mismatched’ hosts (Chapter 5). In the field, specialization manifests as a skew in prevalence towards the matched hosts, and – at least for *A. rigaudi* – an inability to persist in populations that do not contain the matched host (Chapter 3).

A mix of specialist and generalist traits

Specialization is often presented as a dichotomy: specialists, whose fitness is high or null for different hosts, versus generalists, who generally have intermediate fitness (Poulin 2007 chap. 3, Schmid-Hempel 2011 chap. 7, Leggett et al. 2013). *A. rigaudi* and *E. artemiae* fall into a gray zone between these categories, being neither true specialists – they can exploit both hosts –, nor true generalists – their fitness is asymmetrical. Their intermediate phenotypes make these species particularly useful to study the evolution of specialization, as we can examine the factors that push them towards one end or another of the spectrum.

Interestingly, the specialism gray zone extends to the individual traits that determine the fitness of *A. rigaudi* and *E. artemiae* (Chapter 5). Were we to classify the two parasites by their rates of spore production, they would be rated robust specialists. If classified by infectivity, in contrast, *A. rigaudi* would be rated a true generalist, and *E. artemiae* an evolvable specialist. A practical consequence of this trait-dependent specificity is that infectivity may not be a good indication of overall parasite specialization. This is made abundantly clear by the example of *A. rigaudi*, which is able to infect *A. franciscana* (almost) as well as *A. parthenogenetica* (Chapters 3-5), yet is unable to persist in pure *A. franciscana* populations (Chapter 3, Lange et al. 2015) because its fitness is too low (Chapter 4).

Clues to the mystery of E. artemiae

With the results of Chapter 4 in hand, informed speculation about the origin of *E. artemiae* is possible. As previously suspected (Rode et al. 2013c), *E. artemiae* is better adapted to *A. franciscana* than it is to *A. parthenogenetica*. This result alone is not sufficient to conclude that *A. franciscana* is *E. artemiae*’s ancestral host, because it does not exclude the possibility of a post-invasion host switch from *A. parthenogenetica*. However, exposure to *E. artemiae* triggers severe immunopathology in *A. parthenogenetica*, suggesting that the two do not have a shared evolutionary history (Moret 2003, Hasu et al. 2009, Auld et al. 2013), and therefore that *A. franciscana* is the ancestral host. If so, it remains unclear how *E. artemiae* invaded the Mediterranean. Rode et al. (2013c) reported that direct transfer of infected individuals was

unlikely. When they tested whether *E. artemiae* spores could be transferred alongside *A. franciscana* cysts, the results were negative, indicating that co-introduction of *E. artemiae* spores is (at best) extremely rare. However, Rode et al.'s tests necessarily used fewer cysts than the vast quantity that was introduced into the Old World, so even extremely rare co-introductions of *E. artemiae* may have sufficed to kick start a local population.

Coinfection, the missing puzzle piece

An important aspect of the biology of *A. rigaudi* and *E. artemiae*, which has not yet been addressed, is the effect of coinfection. In the field, the two microsporidians regularly coinfect (Chapter 3), and *A. rigaudi* often shares a host with *F. liguloides* (Rode et al. 2013b). The presence of other parasite species in the host is therefore a recurring biotic challenge.

Coinfecting parasite species may have a variety of antagonistic and synergistic interactions (reviewed in Cox 2001, Read and Taylor 2001, Mideo 2009, Rigaud et al. 2010). Antagonistic reactions can affect the establishment or within-host growth of coinfecting species, as parasites compete for host resources (exploitation competition), trigger cross-reactive immune reactions (apparent competition), or cause direct damage (interference competition) (e.g. Pilarska et al. 2006, Lohr et al. 2010, Ben-Ami et al. 2011, Duncan et al. 2015, Natsopoulou et al. 2015). Parasites with conflicting transmission strategies may also hinder each other's transmission (e.g. Haine et al. 2005). Alternately, different parasite species may facilitate each other by depressing the host's immune system (Cox 2001, Graham 2008). This assortment of possible interactions can prompt a variety of evolutionary responses, including changes in virulence, resource partitioning, and the acquisition of competitive traits (see Choisy and de Roode 2010, Alizon et al. 2013).

Regarding the establishment of infection, field data suggests that none of the various parasites infecting *Artemia* in Aigues-Mortes strongly facilitates or inhibits the others. *A. rigaudi* and *E. artemiae* are positively associated in *A. franciscana* (Chapter 3), a result that could indeed be due to facilitation, but could also be caused by an aggregation of parasites in older individuals (Rode et al. 2013c). *A. rigaudi* and *E. artemiae* infect *A. parthenogenetica* independently (Chapter 3), and neither microsporidian is positively or negatively associated with *F. liguloides* (Rode et al. 2013b).

Once *A. rigaudi* and *E. artemiae* have established themselves in a shared host, however, it seems reasonable to expect an antagonistic interaction. The high death rates caused by *A. rigaudi* in *A. franciscana*, for example, are likely to be disadvantageous for *E. artemiae*, and could select for faster exploitation in the latter (Chapter 4). Furthermore, both microsporidians undergo density-dependent regulation when infecting their matched hosts, with peak densities imposed by resource depletion or the host's immune response (discussed in Chapter 4). If *A. rigaudi* and *E. artemiae* exploit overlapping host resources – e.g. uninfected gut epithelium cells – or share vulnerability to the immune system, they may be cross-regulated in coinfecting hosts (cf. Bruce

et al. 2000). Such cross-regulation would favor the fastest-growing parasite, a situation which could select for higher rates of exploitation (de Roode et al. 2005, Ben-Ami et al. 2008). However, the competition is rigged: each microsporidian has a much higher growth rate in its matched host (Chapter 4), and should easily be able to outcompete the mismatched parasite. This asymmetry may be more likely to reinforce specialization (via niche partitioning, Karvonen et al. 2006, Poulin 2007 chap. 8) than to select for competitiveness.

It is hard to guess what kind of interaction, if any, occurs between the microsporidians and the dominant helminth *F. liguloides*. The two groups have different modes of transmission, which may lead to conflicts of interest. *F. liguloides*' trophic transmission route is dependent on the survival of its *Artemia* host until it can be predated by a suitable final host (Amat et al. 1991a), so it is probably ill served by *A. rigaudi*'s lifespan-shortening effects (Chapter 4). In turn, the microsporidians should have no interest in being consumed by a flamingo (discussed by Rode et al. 2013b). Within the host, Graham (2008) showed that helminths can regulate the population size of coinfecting microparasites from the top down, if they boost the immune system, or from the bottom up, if they compete for the same resources. Our understanding of these factors is rudimentary for all three parasites (though there is some information for *F. liguloides*, Amat et al. 1991a, Varo et al. 2000, Sánchez et al. 2009, 2013b), so we cannot predict the type of within-host interaction that is likely to occur. However, we do note that *E. artemiae*, when infecting its matched host *A. franciscana*, causes a reduction in offspring production. The use of the host's reproductive resources infringes on the territory of the castrating *F. liguloides*, which is in the process of colonizing this host (Appendix 2). *E. artemiae*, who until recently was relatively isolated from *F. liguloides*, may therefore find itself competing with an imposing adversary.

Down a level: genotype effects

Finally, an important avenue for future research in the *Artemia* host-parasite system is to study hosts and parasites on a genotype level. Genotype effects are ubiquitous and important in host-parasite species: parasite genotypes differ in host range, life history, and virulence; host genotypes differ in susceptibility and tolerance; and interactions between the two can drive antagonistic coevolution (Carius et al. 2001, Buckling and Rainey 2002, Webster et al. 2004, Decaestecker et al. 2007, Gaba and Ebert 2009, Luijckx et al. 2011, among many others). At this time, very little is known about genotype-level effects in the *Artemia* system; what we do know comes exclusively from the study of different *A. parthenogenetica* clones. Thus, we know that the susceptibility to *E. artemiae*, and possibly *A. rigaudi*, varies across clones (Rode et al. 2013b, Chapter 3). In addition, though all *A. parthenogenetica* clones have the same probability of infection with *F. liguloides*, some clones are less susceptible to its behavioral manipulation (Rode et al. 2013b). To probe further into these genotype effects, and explore potential interactions between host and parasite genotypes, a key next step will be the development of molecular markers for *A. rigaudi*, *E. artemiae*, and *F. liguloides*.

CONCLUSION

CONCLUSION

Artemia and the big bad biotic factors

Artemia are extremophiles that thrive in unstable environments with prohibitive salinities (Cole and Brown 1967, Van Stappen 2002). Unsurprisingly, therefore, their evolution is usually seen as driven by the abiotic environment (Van Stappen 2002, Lindholm 2014), and in this respect they have indeed been remarkably successful. *Artemia* possess astounding physiological plasticity, allowing them to deal with a wide range of physicochemical conditions, as well as the ability to escape harsh conditions as dormant cysts (Clegg and Trotman 2002).

In contrast, this dissertation and other recent studies highlight the importance of the biotic challenges facing *Artemia*. Both *A. franciscana* and *A. parthenogenetica* must cope with parasite manipulation (Rode et al. 2013b), extensive parasite-induced reductions in survival and fecundity (Chapter 4, Amat et al. 1991a), and the constraining of their ecological niche by their microbial symbionts (Nougue et al. 2015). In areas invaded by *A. franciscana*, the *Artemia* also compete for food (Browne 1980, Browne and Halanych 1989, Lenz and Browne 1991) and through reproductive interference (Chapter 1). The magnitude of these negative effects, coupled with the prevalence of these interactions, must impose severe demographic costs and selective pressures on the *Artemia* population.

The classic view that *Artemia* ‘escaped’ from biotic selection pressures by adapting to harsh abiotic environments (Van Stappen 2002, Lindholm 2014) is thus an oversimplification: predation is indeed reduced in the saltern environment, but there are plenty of other interspecific interactions to take up the slack.

Novel interactions and the ease of adaptation

It is likely that the introduction of *A. franciscana* to the Mediterranean did not cause dramatic changes in its abiotic environment. For *Artemia*, the abiotic environment is determined by three key factors (Clegg and Trotman 2002): salinity, which is handled plastically; ionic concentration, which is similar in the ancestral and invasive range (especially in commercial salterns, Cole and Brown 1967); and temperature. The temperature range in Aigues-Mortes is respectively more and less seasonal than those of the San Francisco Bay and of the Great Salt Lake, the source locations of introduced *A. franciscana* cysts (min. to max. monthly temperatures: 8 to 21°C in San Francisco Bay, -3 to 32°C in Great Salt Lake, 2 to 28°C in Aigues-Mortes; Association Infoclimat 2001, US Climate Data 2016). Aigues-Mortes may therefore have periods of sub- or super-optimal temperature for *A. franciscana*, but the invaders should be able to bridge these periods by producing dormant cysts.

In contrast, the introduction of *A. franciscana* to Aigues-Mortes caused a dramatic change in its biotic environment, and in the biotic environment of the native species. Both *Artemia* species were suddenly exposed to a congeneric competitor, *A. franciscana* was released from most of its

ancestral parasites and exposed to Mediterranean parasites, and these came into contact with a new potential host. What can we learn from these novel interspecific interactions?

Surprisingly, we have learned that many of the interactions that developed in the wake of *A. franciscana* are characterized by enduring maladaptation. This is exemplified by *A. franciscana*'s failure to lose its maladaptive sex allocation behavior (Chapter 1), by the 40-year delay between the invasion of *A. franciscana* and the first *F. liguloides* infections observed in this host in Aigues-Mortes (Appendix 2), and by the disproportionate costs incurred by *Artemia* hosts resisting their novel microsporidian parasites (Chapter 4). Such a degree of persistent maladaptation is rather unexpected: in the past decades, it has become clear that adaptive evolution can occur within tens or hundreds of generations (Thompson 1998, Reznick and Ghalambor 2001b), and that such rapid evolution is often triggered by species invasions (Strauss et al. 2006, Sax et al. 2007, Buswell et al. 2011). As with all evolutionary processes, however, adaptation is only one side of the coin (Coyne 2009, Shine 2012). The failure of *A. franciscana*, *A. parthenogenetica*, and their parasites to adapt to their changing biotic environment could be due to a variety of factors, including, but not limited to: a lack of standing genetic variation, caused by bottlenecking (Nei et al. 1975, though see Roman and Darling 2007, Dlugosch and Parker 2008); a lack of mutational genetic variation, due to small population size (Crow and Kimura 1970) or strong trade-offs (Ravigne et al. 2009, Martin and Lenormand 2015); a slowed sweep, due to large population size (Crow and Kimura 1970); and gene flow from nearby unselected populations (Vermeij 1982, Santon and Galen 1997, Lenormand 2002, Benmayor et al. 2009, Cenzer 2016).

Some final thoughts

Over the course of my PhD, I investigated a variety of ecological and evolutionary processes, including sex allocation, mate guarding, disease dynamics, and the evolution of multi-host parasites. In and of themselves, these studies produced a variety of interesting results. Taken together, they have provided some additional insights into the ecological weight and evolutionary trajectory of biotic interactions. Finally, of all the interactions pictured in Fig. 2 of the Introduction, only a handful now remain unexplored. We have gained considerable insights into the major selection pressures operating in the Aigues-Mortes ecosystem, insights which – in the way of science – will drive more questions, new research, and ultimately a greater understanding of the world around us.

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APPENDICES

APPENDIX 1

SURVIVAL OF MICROSPORIDIAN SPORES AT COLD TEMPERATURES & SPORE DORMANCY

CONTEXT

The transmission stages of horizontally transmitted parasites must be able to withstand the stressors of the external environment, including temperature, UV radiation, and drought, until they infect a new host. For parasites whose host is seasonally absent, this ability is particularly important. In general, such parasites should adapt to the precise stressors incurred by their ecology, and adjust their resistance to the length of the hostless period (Maddox 2002, Goertz and Hoch 2008).

The *Artemia*-microsporidian system of Aigues-Mortes is characterized by distinct seasonality, including seasonal changes in host availability (see Chapter 3). In winter, *A. parthenogenetica* is entirely absent (Lievens et al. 2016), and *A. franciscana* densities are low (Rullman et al. unpublished data). Their horizontally transmitted microsporidian parasites should therefore have some ability to survive in the environment under winter conditions. We expect this to be particularly true for *A. rigaudi*, because it depends on *A. parthenogenetica* to persist in the host community (Chapter 3). The preferred host of *E. artemiae*, *A. franciscana*, is present in winter, but its low densities may still promote extended tolerance of environmental conditions for this parasite. Furthermore, in other areas of *E. artemiae*'s range, its *A. franciscana* hosts are absent during the winter (Gliwicz et al. 1995, Rode et al. 2013c, E. J. P. Lievens unpublished data).

Here, I present the results of an experiment designed to measure *A. rigaudi* and *E. artemiae*'s ability to withstand the dominant factor determining winter conditions in the natural system: cold temperature.

METHODS

In this experiment, we collected spores from laboratory stock populations of *A. rigaudi* and *E. artemiae* at two-week intervals over a period of 16 weeks, and stored these samples at 4°C. We then tested their viability through experimental infections of *Artemia* hosts.

The stocks of *A. rigaudi* and *E. artemiae* used for this experiment were similar to those described in Chapter 4: we combined infected *Artemia* from Aigues-Mortes field populations that were heavily infected with either *A. rigaudi* or *E. artemiae*. The infected hosts were kept in 5 L Gilson-type separating funnels, filled to roughly half capacity with saline medium (see

below). In this setup, the infected hosts' feces (containing spores) settle into the collection tube of the funnel, forming a fecal pellet which is easy to collect.

Once every two weeks over a 16-week period, we collected the fecal pellet produced in the microsporidian stock populations. Feces were allowed to accumulate for one week prior to collection. Collection involved the simple opening of the funnel's tap; the feces (in their original saline medium) were then transferred to 50 mL Falcon tubes, and stored at 4°C until testing. 4°C is a representative winter temperature for Southern French salterns (Gabrion et al. 1982). The sample collected at the end of the collection period (0 weeks before testing) was not stored, but used immediately for the viability test. There was no collection for either microsporidian 10 weeks before testing, and for *E. artemiae* 12 weeks before testing.

At the end of the collection period, we tested the viability of the stored samples by experimentally infecting *Artemia* hosts. For each sample, we exposed three groups of 10 uninfected *Artemia* to 10 000 spores each. Exposure happened in a small volume (30 mL per group), to maximize contact with spores. After two days, we increased the volume to 150 mL, and allowed the infection to incubate for an additional 12 days (enough time to allow within-group infections, Rode et al. 2013a). We then sacrificed the surviving individuals, and tested them for the presence of microsporidian DNA by PCR (following Rode et al. 2013a). The experimental *Artemia* were a mixed population of *A. franciscana* and *A. parthenogenetica* juveniles, hatched from dormant cysts sampled from the saltern of Aigues-Mortes and stored in dry condition at 4 °C. Cysts were hatched following the protocol described by Lievens et al. (2016) and raised in parasite-free conditions in the lab (see below).

The spore concentration of the fecal samples was measured on the basis of a 990 µL subsample. The subsample was first stained with 10 µL 1X Calcofluor White Stain (18909 Sigma-Aldrich, USA), then rinsed twice. To rinse the sample, we centrifuged it for 15 min at 10 000 g, replaced 900 µL of the supernate with fresh deionized water, and vortexed the whole. We then added 429 µL of pure Ludox (Sigma-Aldrich, USA) and vortexed well, resulting in a homogeneous 30 % Ludox sample. Each sample was centrifuged for 15 min at 10 000 g, after which 1 000 µL of the supernate was removed. To rinse the sample, we added 750 µL deionized water, centrifuged for 4 min at 8 000 g, and removed 900 µL of the supernate. The remaining 100 µL contained the purified and stained spores. Note that unlike the measurements of concentration described in Chapters 4 and 5, these fecal samples were not homogenized prior to the measurement; some error is therefore likely.

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: groups were fed 5 mL [0.68×10^9 cells/L] *T. chuii*

solution on the second day, and 2 mL [3.4×10^9 cells/L] *T. chuii* solution every two days afterwards.

RESULTS

Both *A. rigaudi* and *E. artemiae* could be stored at 4°C for several weeks without loss of viability (Fig. 1). *A. rigaudi* spores remained viable during at least 16 weeks of storage at cold temperatures (with the exception of the sample collected 8 weeks before testing, which appears to have been defective). *E. artemiae* spores could only be stored for 6–8 weeks before losing their ability to infect new hosts.

Surprisingly, the fecal samples used immediately after collection were much less infective than the others (0 weeks at 4°C, Fig. 1). Because this was true for both the *A. rigaudi* and the *E. artemiae* samples, we suspected that this pattern was determined by a shared environmental factor. The ‘0 weeks’ samples differed from the others because they had not undergone the ‘cold storage’ treatment; we therefore tested whether cold storage could restore their ability to infect. After one month of storage at 4°C, these spores did indeed become infective (blue points in Fig. 1); a control sample stored for one month at ambient temperature did not (orange points in Fig. 1).

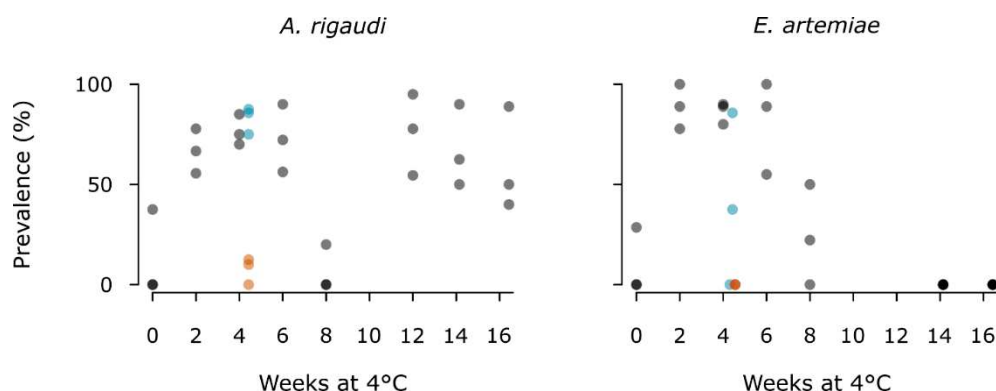


Figure 1. Infection success of *A. rigaudi* and *E. artemiae* after storage at 4°C. At each time point, the set of three points represents one fecal sample, tested in three replicate host groups. The samples tested immediately after collection (0 weeks at 4°C) were split and stored at 4 or 23°C for one month; the results of their subsequent retesting are shown in blue and orange, respectively. Overlapping points shade to black or dark blue/orange.

DISCUSSION & PERSPECTIVES

A. rigaudi and *E. artemiae*’s tolerance of cold temperatures corresponded well to our expectations based on the seasonal ecology of the system. The spores of *A. rigaudi*, whose primary host *A. parthenogenetica* is absent for roughly four months in winter (Lievens et al. 2016), were able to tolerate storage at 4°C for at least 16 weeks (≈ 3.7 months). The spores of

E. artemiae, whose primary host *A. franciscana* is less seasonal, lost their ability to infect more quickly.

A particularly intriguing result of this experiment was that spores collected after one week of sedimentation were not infective, but clearly viable – as shown by their regained infectivity after exposure to cold temperatures. We know from other studies that *A. rigaudi* and *E. artemiae* spores do not require a maturation period; they are infective immediately after release into the gut lumen (Rode et al. 2013a, Chapters 3 and 4). We have no reason to suppose that the ‘0 weeks at 4°C’ samples were different. Therefore, we conclude that previously-infective spores become non-infective, or dormant, after sedimentation in the Gilson funnel, and that this dormancy can subsequently be removed by exposure to cold temperatures. Some previous research in *Brachiola algerae* has indicated that spores can be ‘inactivated’ and ‘activated’ by certain environmental conditions (Undeen and Avery 1988, Undeen and Solter 1996, *Brachiola algerae* was then *Nosema algerae*, Vossbrinck and Debrunner-Vossbrinck 2005). Alternately, *A. rigaudi* and *E. artemiae* could produce two spore types, one short-lived and one dormant (up to four spore types have been described in other microsporidian species for different aspects of transmission, Becnel et al. 1989, Agnew et al. 2003).

Attempts to replicate the ‘dormancy and revival’ of spores have thus far had mixed success. However, for various reasons, the replication protocols all diverged subtly from the original protocol. A true replication is pending; meanwhile, the results of the quasi-replication experiments suggest that the induction of dormancy may be sensitive to the size of the fecal pellet (possibly related to changes in light or anoxic conditions) and to homogenization after collection.

APPENDIX 2

COLONIZATION OF *ARTEMIA FRANCISCANA* BY THE CESTODE *FLAMINGOLEPIS LIGULOIDES*

CONTEXT

The cestode *Flamingolepis liguloides* is a parasite of the Greater Flamingo, *Phoenicopterus roseus* (Robert and Gabrion 1991), whose larval stage infects the genus *Artemia*. The cestode's range stretches throughout the Mediterranean and into Kazakhstan, where it can regularly be found infecting *Artemia parthenogenetica* and *Artemia salina* (a sexual species native to the Mediterranean that has been severely out-competed by *Artemia franciscana*, Amat et al. 2005) at high prevalence (Table 1).

Although *A. franciscana* has been present in the Mediterranean region for several decades (Amat et al. 2005), *F. liguloides* has only rarely been detected in this host, and when found its prevalence has been very low (Table 1). This has been widely interpreted as a sign of host resistance, a conclusion which is supported by the observation of dead larval cestodes in *A. franciscana* (Georgiev et al. 2014, Redón et al. 2015a). Because infection with *F. liguloides* has severe costs for host fitness (through castration, Amat et al. 1991a, Rode et al. 2013b, Redón et al. 2015a), *A. franciscana*'s resistance to it provides this invasive species with a considerable advantage (enemy release, Sánchez et al. 2012).

Here, I report that since 2011, live *F. liguloides* larvae have regularly been detected at moderate to high prevalences in French *A. franciscana*. These observations strongly suggest that the host range of this cestode species is in the process of expanding.

METHODS

We collected live *Artemia* from Aigues-Mortes at various times between October 2013 and June 2014, and characterized their levels of infection with *F. liguloides*. A random subset of adult *Artemia* was selected immediately after sampling; each individual in the subset was then identified (*A. franciscana* or *A. parthenogenetica*) and checked for the presence of *F. liguloides* larvae under a binocular microscope.

Table 1. Published prevalence data for *F. liguloides*. Prevalence values separated by ‘-’ indicate minimum and maximum prevalences; ‘~’ indicates a mean over the sampling period. Blank cells indicate the host was locally absent. *A.p.*, *A. parthenogenetica*; *A.f.*, *A. franciscana*; *A. s.*, *A. salina*.

Location	Sampling date	Prevalence			Ref.
		in <i>A. p.</i>	in <i>A. f.</i>	in <i>A. s.</i>	
Algeria					
Chott Marouane	winter 2000-2001			10-33 %	10
Sebkha Ez-Zemoul	spring 2003			10-33 %	10
France					
Aigues-Mortes	Oct. 1988	7 %			1
"	July - Dec. 2008	~ 67 %	0 %		2
"	May 2011	37 - 83 %	0 %		3
Berre	Nov. 1988	1 %			1
Fangassier	Oct. 1988	10 %			1
Fos-sur-Mer	Nov. 1988	27 %			1
Pesquiers	Nov. 1988	54 %			1
Portugal					
Castro Marim	Aug. 2005		1 %		4
Spain					
Bonmati	Oct. 1988	18 %			12
Cerillos	Aug. 2005			77 %	4
"	Oct. 2006 - Oct. 2007			23 - 64 %	5
Ebro Delta	Jan. 2009 - Jan. 2010		~ 0.8 %	~ 18 %	6
La Mata	Oct. 2007	35 %			7
La Tapa	Dec. 2006 - Sept. 2007		0.2 - 2 %		8
Las Animas	Oct. 2005		0 %		4
Nuestra Señora del Rocio	Aug. 2005	67 %			4
Odiel	Aug. 2005	45 %			4
"	Oct. 2002 - Aug. 2003	2 - 43 %			5
Portuguesas	Aug. 2005	54 %			4
Santa Barbara	Oct. 2005		0 %		4

References: 1. Thiéry et al. 1990; 2. Sánchez et al. 2012; 3. Rode et al. 2013; 4. Georgiev et al. 2007; 5. Sánchez et al. 2013; 6. Redón et al. 2015; 7. Redón et al. 2011; 8. Georgiev et al. 2014; 10. Amarouayache et al. 2009; 12. Amat et al. 1991b.

Excluded due to unspecified *Artemia* species: Maksimova 1973, 1989 (Lake Tengyz, Kazakhstan, reported as *F. dolguschini*), Gabrion et al. 1982 (Camargue, France), Robert and Gabrion 1991 (Salin de Giraud, France), Mura 1995 (Sardinia, Italy).

RESULTS

The prevalence of *F. liguloides* varied between 0 and 100%, and tended to be higher in *A. parthenogenetica* (Fig. 1). However, *F. liguloides* infected over half of all adult *A. franciscana* in two samples, and regularly reached prevalences well above the published maximum of 2% in this host (Fig. 1).

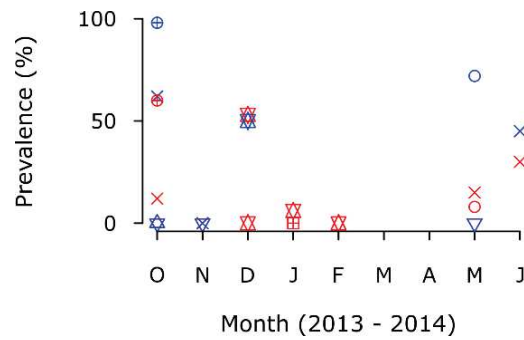


Figure 1. Prevalence of the cestode *F. liguloides* in *A. franciscana* (red) and *A. parthenogenetica* (blue) adults from October 2013 to June 2014. Different symbols represent the collection sites.

DISCUSSION & PERSPECTIVES

Until at least 2011, *F. liguloides* was so rare in *A. franciscana* that it was never detected infecting this host in Aigues-Mortes (Table 1). By the end of 2013, however, this situation had changed dramatically, with *F. liguloides* infecting over half of the adult *A. franciscana* in some samples. A similar trend has been detected in various Spanish populations (M. Sánchez, personal communication), precluding any possibility that this difference is due to a change in the local populations of *A. franciscana*. Instead, the increase in prevalence is very likely due to a sudden increase in the parasite's infectivity. In other words, *F. liguloides* has bypassed a barrier which kept it from exploiting *A. franciscana*, and is now extending its host range. The simultaneous occurrence of this range expansion in France and Spain is likely due to flamingo-mediated dispersal of *F. liguloides* throughout the Mediterranean (Balkiz et al. 2010). The opportunity to track host switching in real time is rare, so this is an exciting and promising finding. It also suggests that *A. franciscana*'s advantage through enemy release will soon decrease, as least as regards this highly prevalent castrating parasite.

APPENDIX 3

RELATIONSHIPS BETWEEN FITNESS, INFECTIVITY, VIRULENCE, AND SPORE PRODUCTION IN THE SERIAL PASSAGE EXPERIMENT

CONTEXT

In the serial passage experiment described in Chapter 5, selection for specialism or generalism did not affect the mean fitness attained by *A. rigaudi* and *E. artemiae* in their matched hosts. However, there was considerable variation in fitness among selection lines, and lines also varied in their infectivity, virulence, and spore production. Generating these lines provided an opportunity to study the genetic correlations between infectivity, virulence, and spore production in *A. rigaudi* and *E. artemiae* (Gandon 2004, Rigaud et al. 2010, Leggett et al. 2013), and indeed to test for the existence of optimal virulence (Alizon et al. 2009).

METHODS

Using the data produced in Chapter 5 for *A. rigaudi* assayed on *A. parthenogenetica* and *E. artemiae* assayed on *A. franciscana*, we investigated the relationships between infectivity, virulence, and spore production, as well as the relationship between virulence and fitness. For each pairwise comparison, we fit generalized linear models with a null, linear, quadratic, or square root function of the predictor. All models were weighted by the number of infected individuals in the long-term assay, because this determined the precision of the virulence and spore production estimates. The revived *A. rigaudi* line was always included, because it did not differ from the surviving lines. In contrast, revived lines were always excluded for *A. franciscana-E. artemiae*, because storage at 4°C was found to affect the infectivity and fitness of this parasite. We did make one exception to this rule, to compare the virulence and rate of spore production of *E. artemiae*. Because neither factor was affected by revival, we ran an additional analysis that did include revived lines. The line *A. rigaudi*/‘Single host *A. p.*’ – Replicate 1 could not be included in these analyses, because none of the hosts in the final assays were infected. Model comparison was done using the corrected AIC (Hurvich and Tsai 1989).

RESULTS

For *A. rigaudi* infecting *A. parthenogenetica*, spore production was independent of virulence (Fig. 1, Table 1), as was the overall fitness (Fig. 2, Table 1). Infectivity was slightly higher at low virulence, but this effect was qualitatively minor (Fig. 1, Table 1). In contrast, the most

infective lines had intermediate rates of spore production at the time of passing (Fig. 1, Table 1).

In contrast, there were clear relationships between infectivity, virulence, spore production, and fitness for *E. artemiae* assayed in *A. franciscana*. Intermediately-virulent lines had the highest rates of spore production and infectivity (Fig. 1, Table 1) and consequently the highest fitness, which was a composite measure of the two (Fig. 2, Table 1). Infectivity and spore production were positively related (Fig. 1, Table 1).

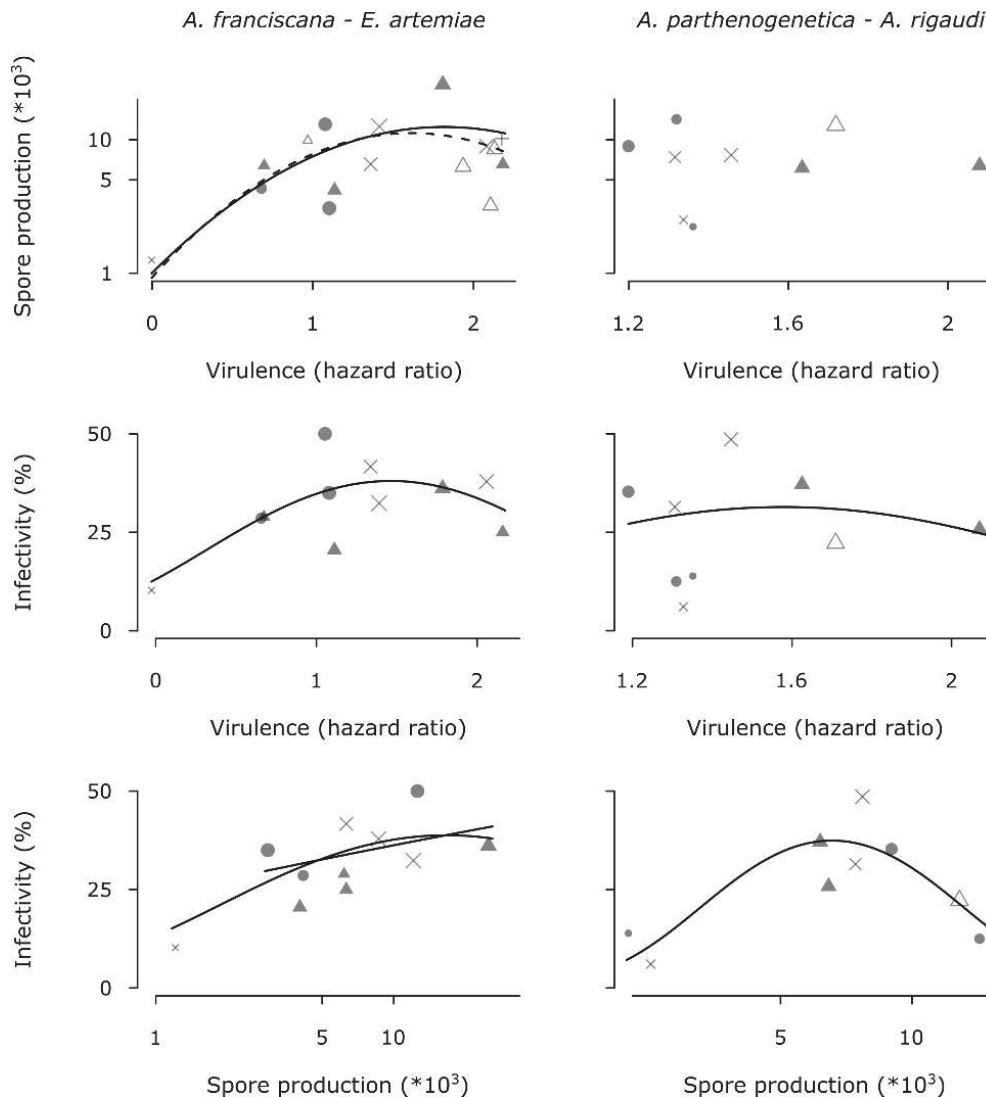


Figure 1. Relationships between infectivity, virulence, and spore production of *A. rigaudi* and *E. artemiae* when assayed on their matched hosts. Infectivity is the prevalence of infection in the short-term assay; virulence is the hazard ratio of infected hosts compared to unexposed controls; spore production is the number of spores produced per infected host at the time of passing (\ln scale). Symbols represent the selection regime (triangles, 'Single host *A. f.*'; circles, 'Single host *A. p.*'; crosses, 'Alternating hosts'); empty symbols represent revived lines; the area of each data point indicates its statistical weight. Lines trace the best model predictions. In the bottom left graph, the fit was sensitive to the presence of the outlier; the best fits are shown for models with and without this data point. For *A. franciscana*-*E. artemiae*, revived lines could be included when

comparing virulence and spore production (top left); the dashed line shows the best fit of this additional model.

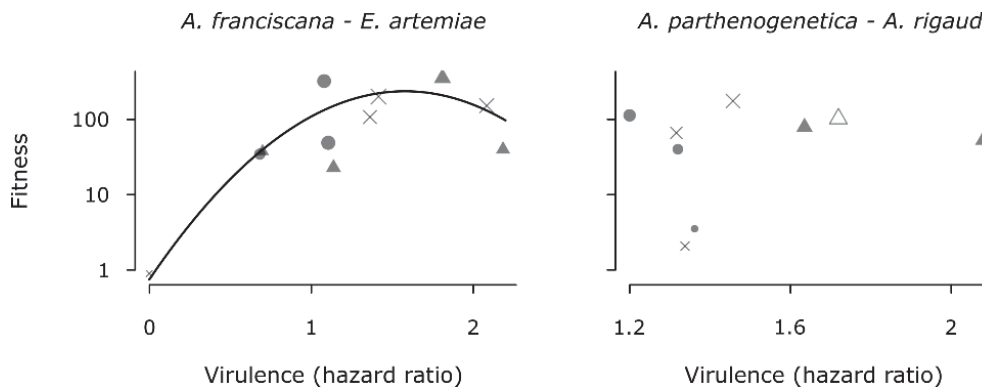


Figure 2. Relationship between virulence and fitness of *A. rigaudi* and *E. artemiae* when assayed on their matched hosts. Fitness is the projected number of infections at the time of passing (\ln scale); virulence is the hazard ratio compared to unexposed controls. Symbols represent the selection regime (triangles, 'Single host *A. f.*'; circles, 'Single host *A. p.*'; crosses, 'Alternating hosts'); empty symbols represent revived lines (excluded for *A. franciscana*-*E. artemiae*). The area of each data point indicates its statistical weight. Lines trace the best model predictions.

DISCUSSION & PERSPECTIVES

In both *A. rigaudi* and *E. artemiae*, infectivity and spore production may be functionally linked by the process of autoinfection. As discussed in Chapter 4, infection most likely spreads within the host when spores, once released into the gut lumen, germinate and infect new cells (Agnew et al. 2003, Refardt and Ebert 2006). This implies that the rate of within-host growth is positively correlated with infectivity. Indeed, if we assume no differences in the rate of spore maturation, infectivity should be the key factor determining within-host growth. This effect, when combined with the different mechanisms of density dependence acting on *A. rigaudi* and *E. artemiae* in their matched hosts (Chapter 4), could produce the different relationships between infectivity and spore production that we see in Fig. 1. For *A. rigaudi* in *A. parthenogenetica*, density dependence limits the within-host population to a certain peak size, which is reached more quickly if the within-host growth rate is higher. Afterwards, the rate of spore production decreases quickly. The observed pattern may reflect the fact that high-infectivity lines have high within-host growth rates, and thus reach their peak transmission rate before day 21, while low-infectivity lines have low within-host growth rates, and have yet to reach their transmission peak at day 21. For *E. artemiae* infecting *A. franciscana*, density dependence operates differently, with higher within-host growth leading to higher spore production peaks. Thus, we could expect high-infectivity lines to have high within-host growth rates, leading directly to higher transmission peaks. Pending more information on host exploitation in these selection lines, these conclusions remain speculative.

A second important result is the quadratic relationship between virulence and spore production, and virulence and infectivity, in *E. artemiae* (Fig. 1), which results in an optimal fitness at intermediate levels of virulence (Fig. 1). The evolution of optimal virulence is a central prediction of parasite evolutionary theory (Alizon et al. 2009), but its empirical support is still rather patchy (e.g. Dwyer et al. 1990, Jensen et al. 2006, Fraser et al. 2007, de Roode et al. 2008, Doumayrou et al. 2012). Optimal virulence is expected to emerge from the existence of an optimal exploitation strategy, which determines both the duration of infection (through virulence and recovery) and the extent of transmission. Classically, models assume a trade-off between virulence and transmission, with transmission rate as a saturating function of virulence. We find this relationship for *E. artemiae*, and indeed very high transmission rates are associated with lower virulence. Interestingly, we find the same relationship between infectivity and virulence, lending support to Alizon & Michalakis' (2015) argument that to understand virulence evolution in parasites who don't have classically direct transmission, fitness must be integrated over the entire life cycle of the parasite. Again, further information on the rate of host exploitation in the selection lines will allow us to disentangle the mechanisms underlying the trade-offs in *E. artemiae*, and may explain why we don't see them in *A. rigaudi*.

Table 1. Model comparison of relationships between fitness and virulence, and between infectivity, virulence and spore production. Only results for the combinations *A. franciscana*-*E. artemiae* and *A. parthenogenetica*-*A. rigaudi* are shown. Fitness is the projected number of infections at the time of passaging (negative binomial); virulence is the hazard ratio of infected hosts compared to unexposed controls; infectivity is the prevalence of infection in the short-term assay (binomial); spore production is the number of spores produced per infected host at the time of passaging (rescaled by 0.01, negative binomial). All models were weighted by the number of infected individuals in the long-term assay, which determined the precision of the estimated fitness, virulence, and spore production. Revived lines were always excluded for *A. franciscana*-*E. artemiae*, and included for *A. parthenogenetica*-*A. rigaudi*.

Pairwise comparison	AICc	Δ AICc	w	outlier removed (<i>A. f.-E. a.</i>)		
				AICc	Δ AICc	w
Fitness ~ virulence						
<i>A. franciscana</i> infected with <i>E. artemiae</i>						
~ hazard ratio + hazard ratio ²	2610.6	0	1.00	2599.5	0	1.00
~ square root(hazard ratio)	2693.1	82.5	0.00	2662.0	62.5	0.00
~ hazard ratio	2717.6	107.0	0.00	2668.3	68.8	0.00
~ 1	2743.6	133.0	0.00	2683.1	83.6	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>						
~ 1	1801.1	0	0.73			
~ hazard ratio + hazard ratio ²	1805.0	3.9	0.10			
~ hazard ratio	1805.4	4.3	0.09			
~ square root(hazard ratio)	1805.6	4.5	0.08			
Spore production ~ virulence						
<i>A. franciscana</i> infected with <i>E. artemiae</i> (results including revived lines were qualitatively equivalent)						
~ hazard ratio + hazard ratio ²	2369.8	0	1.00	2338.6	0	1.00
~ square root(hazard ratio)	2386.4	16.6	0.00	2356.8	18.2	0.00
~ hazard ratio	2402.4	32.6	0.00	2363.2	24.6	0.00
~ 1	2468.1	98.3	0.00	2409.3	70.7	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>						
~ 1	1651.7	0	0.82			
~ hazard ratio	1656.2	4.5	0.09			
~ square root(hazard ratio)	1656.2	4.5	0.09			
~ hazard ratio + hazard ratio ²	1660.5	8.8	0.01			
Infectivity ~ virulence						
<i>A. franciscana</i> infected with <i>E. artemiae</i>						
~ hazard ratio + hazard ratio ²	1109.7	0	1.00	1092.5	0	1.00
~ square root(hazard ratio)	1160.0	50.3	0.00	1111.8	19.3	0.00
~ hazard ratio	1178.5	68.8	0.00	1112.3	19.8	0.00
~ 1	1186.2	76.5	0.00	1109.3	16.8	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i> : infectivity ~ virulence						
~ hazard ratio + hazard ratio ²	1052.1	0	0.82			
~ 1	1055.9	3.8	0.12			
~ hazard ratio	1058.6	6.5	0.03			
~ square root(hazard ratio)	1058.8	6.7	0.03			
Infectivity ~ spore production						
<i>A. franciscana</i> infected with <i>E. artemiae</i>						
~ spore production + spore production ²	1100.7	0	1.00	1076.9	2.4	0.14
~ square root(spore production)	1112.6	11.9	0.00	1074.5	0	0.47
~ spore production	1116.5	15.8	0.00	1074.9	0.4	0.39
~ 1	1186.2	85.5	0.00	1109.3	34.8	0.00
<i>A. parthenogenetica</i> infected with <i>A. rigaudi</i>						
~ spore production + spore production ²	758.8	0	1.00			
~ square root(spore production)	1044.2	285.4	0.00			
~ spore production	1048.9	290.1	0.00			
~ 1	1055.9	297.1	0.00			

APPENDIX 4:

RESUME DE LA THESE EN FRANÇAIS

CHAPITRE 1 : AJUSTEMENT MALADAPTATIVE DU SEX-RATIO CHEZ LA CRUSTACEE INVASIVE *ARTEMIA FRANCISCANA*

La théorie de l'allocation des sexes est souvent nommée la plus fructueuse de la biologie évolutive, grâce à sa capacité de prédire des observations empiriques. La plupart des sex-ratios naturels peuvent être expliqués par le principe de l'investissement égal entre les sexes, ou dans certains cas par une allocation « extraordinaire ». Des déviations du sex-ratio attendu sont souvent corrélées avec une sélection faible ou un environnement peu prédictible; de vrais cas de sex-ratio aberrant sont rares. Ici, nous présentons un cas d'allocation de sexe maladaptative de longue durée, que nous avons découvert dans des populations invasives de *Artemia franciscana*, une crustacée sexuée. *A. franciscana* a été introduite en France il y a environ 500 générations; depuis, cette espèce a vécu en sympatrie avec une espèce native asexuée, *Artemia parthenogenetica*. On s'attend à ce que *A. franciscana* produise un sex-ratio équilibré, mais chez les *A. franciscana* invasifs, nous avons régulièrement observé des sex-ratios extrêmement biaisés vers des mâles, et ceux corrélés avec la proportion d'asexuées dans la population générale (Fig. 1). Nous avons ensuite prouvé expérimentalement que les *A. franciscana* de l'aire invasive et native surproduisent des fils quand ils sont exposés à un excès de femelles, sans distinguer entre des femelles conspécifiques et hétérospécifiques (Fig. 2). Nous concluons que les *A. franciscana* ajustent le sex-ratio de leurs descendants en fonction du sex-ratio adulte, mais qu'ils sont limités par l'information en présence des femelles asexuées. Leur trait d'ajustement facultatif, qui était probablement adaptatif dans leur aire native, est donc devenu maladaptatif dans l'aire invasive, où des femelles asexuées sont présentes. Malgré cette maladaptation, le trait a persisté sans changement depuis des centaines de générations.

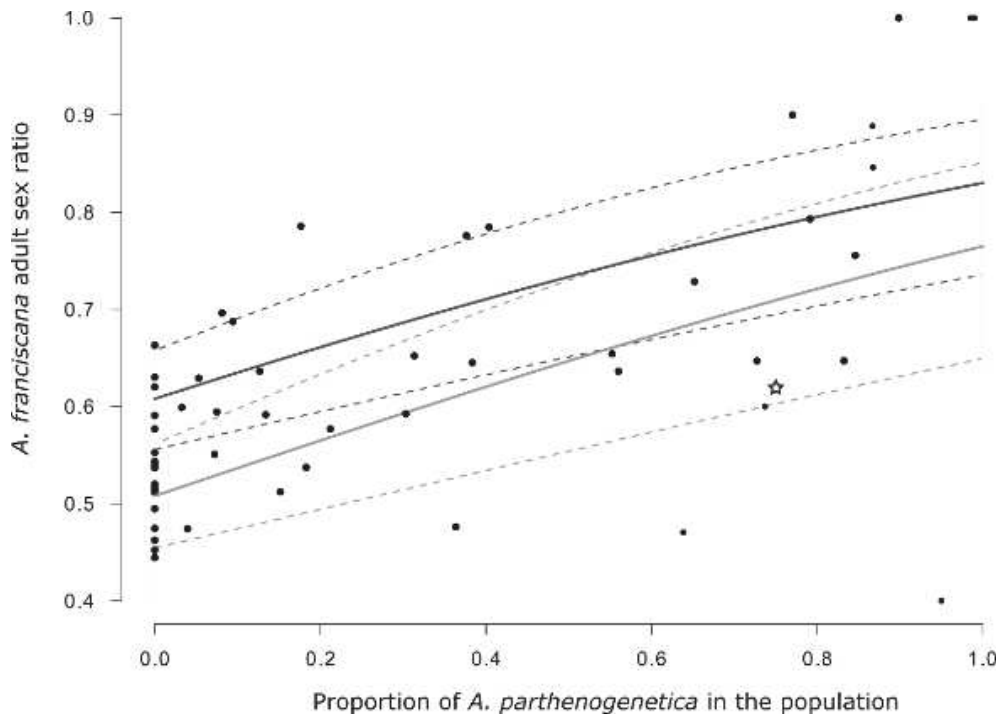


Figure 1. Le sex-ratio des *A. franciscana* en population naturelle augmente en présence des asexuées *A. parthenogenetica*. Le sex-ratio varie aussi pendant l'année ; les courbes représentent les prédictions du meilleur modèle pour décembre (sex-ratios les plus élevés, en gris foncé) et mai (sex-ratios les moins élevés, en gris clair). Les lignes en pointillé représentent les IC 95%. Les échantillons où moins de 20 *A. franciscana* ont été comptés sont représentés par les points petits. L'étoile marque la valeur de notre résultat expérimental (traitement « Invasive range + P », Fig. 2).

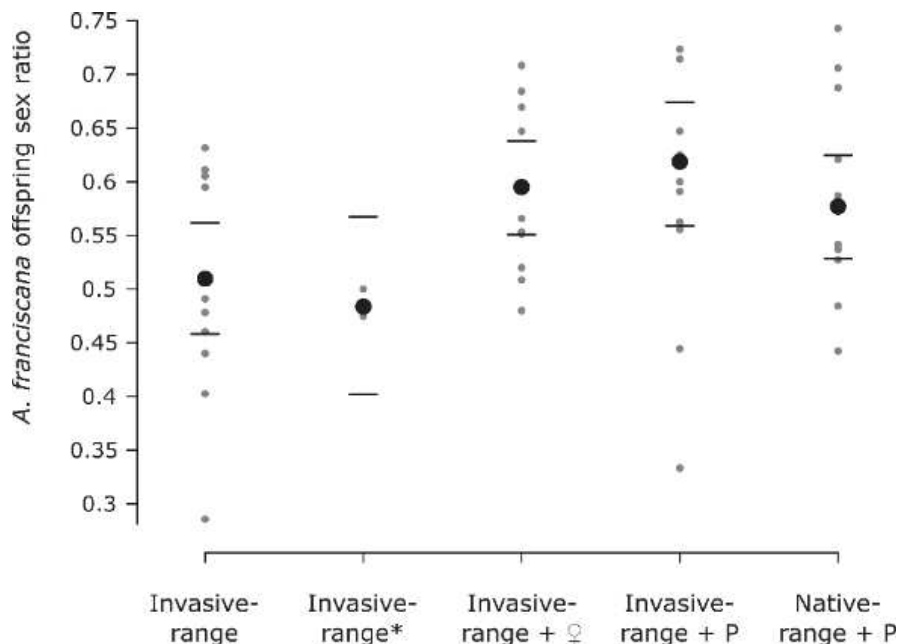


Figure 2. Les *A. franciscana* produisent des sex-ratios biaisés vers les mâles quand ils sont exposés à un sex-ratio adultes biaisé vers les femelles. Ils font ceci indépendamment de leur origine géographique (« aire invasive » vs. « aire native ») et de si le biais femelle est causé par des femelles conspécifiques (« + ♀ ») ou par des asexuées *A. parthenogenetica* (« + P »). Les points gris sont les résultats de chaque réplicat, les points noirs représentent les moyennes par traitement, et les barres horizontales les IC 95% binomial.

CHAPITRE 2 : DISCRIMINATION CONTRE DES FEMELLES HETEROSPECIFIQUES ET NON-RECEPTIVES PENDANT LE GARDIENNAGE DU PARTENAIRE CHEZ *ARTEMIA*

Le gardiennage du partenaire est une stratégie reproductive dans lequel des mâles s'assurent d'une opportunité d'accouplement en gardant des femelles qui ne sont pas encore sexuellement réceptives. Ce comportement de gardiennage est coûteux, donc les mâles devraient préférer garder des femelles conspécifiques et proches à la réceptivité. Nous avons étudié le comportement de gardiennage de *Artemia franciscana*, une crustacée bisexuée, dans son aire invasive dans le Sud de France. Nous avons échantillonné des couples en position de garde et des individus aléatoires à trois sites sur le terrain, dont un qui contenait aussi l'espèce native asexuée *Artemia parthenogenetica*. A tous les sites, des couples avaient une plus grande probabilité de contenir des femelles proches à la réceptivité, bien que le patron ne fût pas entièrement prévisible entre les différents stades reproductifs des femelles (Fig. 3). Là où elles étaient présentes, des femelles *A. parthenogenetica* étaient fortement désélectionnées pour le gardiennage. Nous confirmons aussi des résultats antérieurs qui montraient un appariement assorti par taille chez *Artemia*. Nos résultats soutiennent la prédiction théorique que les mâles devraient préférer des femelles qui maximisent le retour sur leur investissement de gardiennage, et fournissent la première preuve d'une discrimination par espèce chez *Artemia*. Nous discutons des résultats dans le contexte d'un consensus émergent sur le choix de partenaire mâle chez *Artemia*.

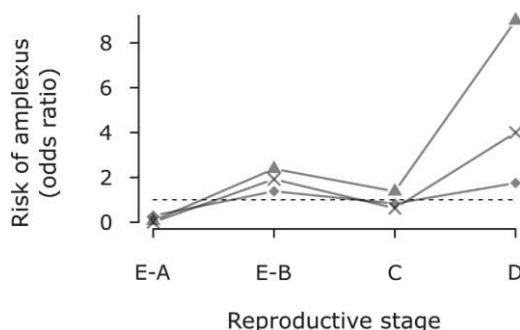


Figure 3. Hasard relatif d'être en amplexus en fonction de l'état reproductif de la femelle. Les stades reproducteurs sont rangés du moment le plus lointain de la réceptivité femelle (à gauche) au moment de réceptivité (à droite). Les symboles montrent les valeurs pour chaque site (diamant, Fangouse; croix, Puit Romain; triangle, Site 9). La ligne en pointillé marque un odds ratio de 1 (amplexus aléatoire). Ces analyses incluent uniquement les individus *A. franciscana*.

CHAPITRE 3 : L'ÉPIDÉMIOLOGIE DE DEUX PARASITES MICROSPORIDIENNES SYMPATRIQUES EST FORMÉE PAR DE LEUR SPÉCIFICITÉ D'HÔTE

L'identification des facteurs qui causent des dynamiques de maladie observées est souvent difficile, surtout dans des systèmes saisonniers et multi-hôtes. Dans ce papier, nous utilisons une combinaison d'expériences et d'un suivi à long terme pour dénouer les effets de la saisonnalité et la spécificité d'hôte sur la prévalence dans une communauté hôte-parasite simple. Nous avons étudié deux microsporidies à transmission horizontale, *Anostracospora rigaudi* et *Enterocytozpora artemiae*, dans leurs hôtes crustacés *Artemia franciscana* et *Artemia parthenogenetica*, dans les salins d'Aigues-Mortes, France. Sur le terrain, la prévalence variait entre 0% et > 90% dans toutes les combinaisons hôte × parasite. *A. rigaudi* était fortement saisonnier, étant très prévalent en été et absent en hiver (Fig. 2). Des modèles statistiques ont montré que cette saisonnalité était conduite par la saisonnalité de l'hôte *A. parthenogenetica*, qui fonctionne comme réservoir ; *A. rigaudi* est incapable de persister dans des communautés d'hôtes qui consistent exclusivement de l'hôte *A. franciscana* (Fig. 3). *E. artemiae* était présent tout au long de l'année, et était plus prévalent dans *A. franciscana* (Fig. 2); nous n'avons pas pu déterminer si ce dernier était aussi un réservoir. En testant expérimentalement l'infectivité des microsporidies à 15°C et 25°C, nous avons confirmé que les dynamiques décrites ici étaient indépendantes des effets de température saisonniers. En général, des propriétés intrinsèques des relations hôte × parasite, et pas le forçage environnemental, déterminaient les différentes dynamiques de maladie des deux microsporidies.

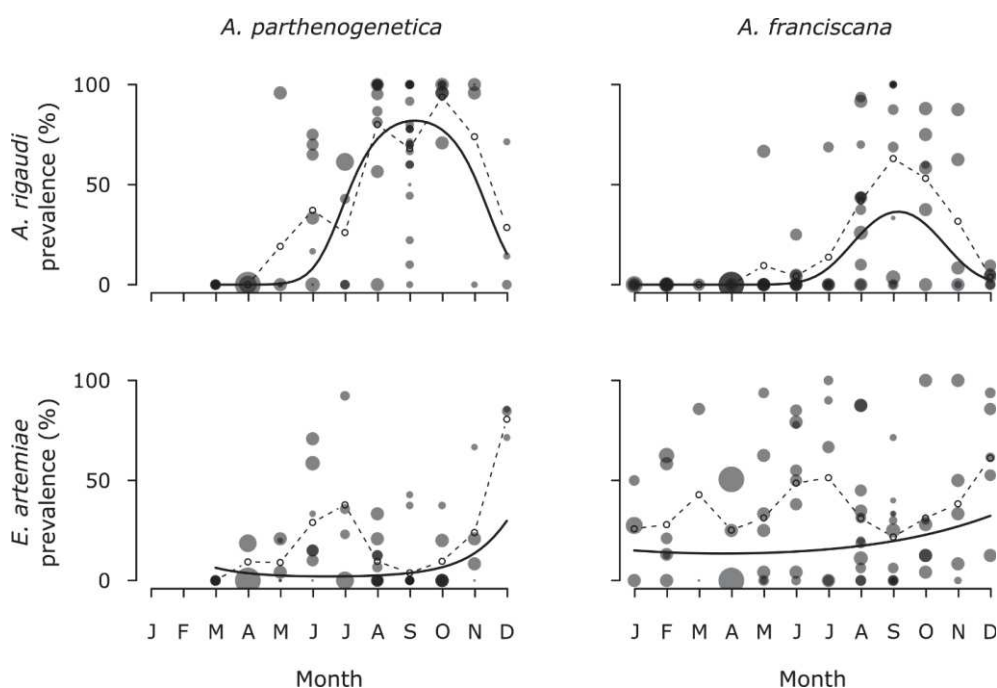


Figure 4. Patrons de prévalences d'*A. rigaudi* (haut) et d'*E. artemiae* (bas) dans *A. parthenogenetica* (gauche) et *A. franciscana* (droite). Les points solides correspondent aux échantillons; la surface de chaque point montre la taille de l'échantillon. Points chevauchants deviennent noirs. Ligne solide: prédictions du meilleur

modèle général; ligne pointillée: prévalences moyennes dans les échantillons. Il n'y a pas de données de prévalence pour *A. parthenogenetica* en janvier et février parce que cette espèce n'est pas présente en hiver.

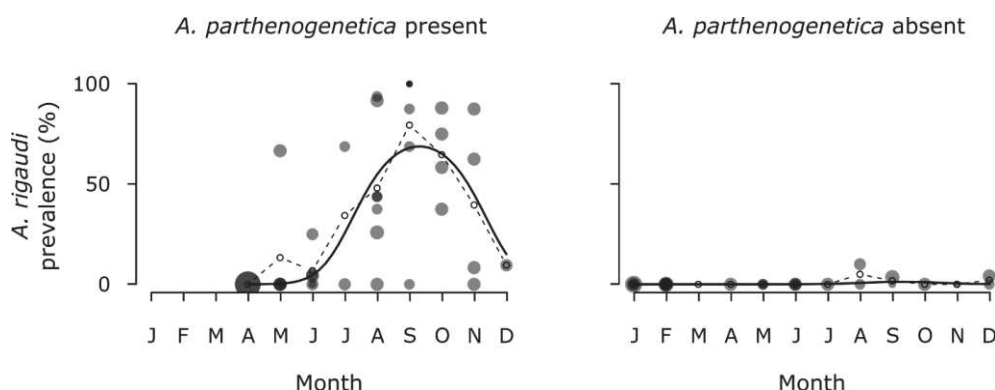


Figure 5. Prévalence d'*A. rigaudi* dans *A. franciscana* quand *A. parthenogenetica* est présente (gauche) ou absente (droite). Ensembles, ces deux graphiques sont équivalents au graphique en haut et droite de Fig. 2. Les points solides correspondent aux échantillons; la surface de chaque point montre la taille de l'échantillon. Points chevauchants deviennent noirs. Ligne solide: prédictions du meilleur modèle général; ligne pointillée: prévalences moyennes dans les échantillons.

CHAPITRE 4 : INFECTIVITE, VIRULENCE ET TRANSMISSION DANS UN SYSTEME DE DEUX HOTES ET DEUX PARASITES

Bien qu'il soit communément reconnu que la majorité des parasites infectent plusieurs espèces d'hôtes, des études empiriques des effets de l'utilisation de multiples hôtes sur l'histoire de vie et la virulence des parasites sont rares. Dans cette étude, nous avons quantifié l'infectivité, la croissance intra-hôte, le taux de transmission, et la virulence de deux parasites à transmission horizontale, les microsporidies *Anostracospora rigaudi* et *Enterocytozpora artemiae*, dans leurs hôtes naturels, les crustacées *Artemia parthenogenetica* et *Artemia franciscana*. Ceci a été fait à l'aide d'infections expérimentales, dont nous avons suivi la production de spores, la croissance, la reproduction, et la survie de chaque hôte infecté. Nos résultats montrent que chaque parasite a une bonne performance sur un des deux espèces d'hôte (*A. rigaudi* sur *A. parthenogenetica*, *E. artemiae* sur *A. franciscana* ; Fig. 7), et que cette spécialisation est associée avec une exploitation sous-optimale, une virulence maladaptative, et des forts coûts de résistance dans l'autre hôte. Notre étude représente l'une des rares contributions empiriques à l'étude de l'évolution des parasites multi-hôtes. Elle met particulièrement en valeur les difficultés de l'adaptation à plusieurs espèces d'hôte.

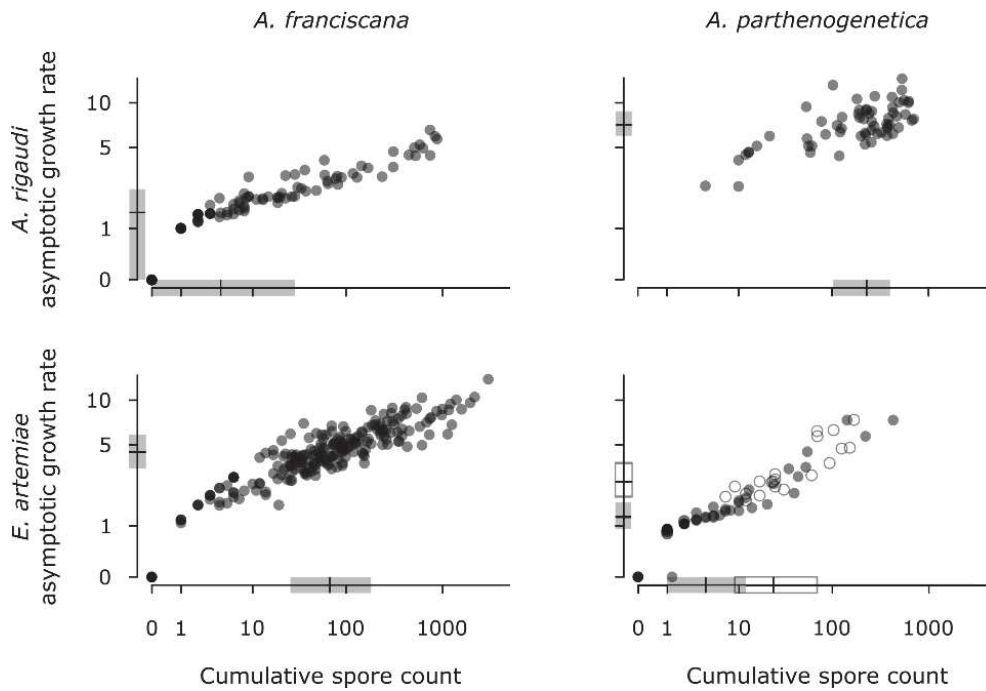


Figure 6. Fitness des infections d'*A. rigaudi* (haut) et *E. artemiae* (bas). Le taux de croissance asymptotique (échelle $\ln + 1$) est donné en fonction de la quantité cumulée de spores produites (échelle $\ln + 1$). La croissance asymptotique devrait être maximisée pendant des épidémies, tandis que la quantité cumulée de spores, comme estimateur de R_0 , devrait être maximisée en conditions endémiques. Les médians, premiers et troisièmes quartiles sont représentés par des box plots sur les axes. Pour *A. parthenogenetica* infectée par *E. artemiae*, les cercles et box plots vides représentent les femelles exposées à une dose de spores plus élevée. Chaque point correspond à un hôte infecté; les points chevauchants deviennent noirs.

CHAPITRE 5 : DEUX PARASITES MICROSPORIDIENNES D'*ARTEMIA* RESTENT SPECIALISTES APRES DES PASSAGES SUCCESSIFS SUR DES HOTES ALTERNATIVES

La théorie de la spécialisation écologique prédit que les populations évoluent vers le spécialisme quand les trade-offs environnementaux sont forts, et vers le généralisme quand ils sont faibles. Dans cette étude, nous avons utilisé une approche d'évolution expérimentale pour étudier l'évolution de la spécialisation parasitaire, et les trade-offs sous-jacents, chez deux parasites sympatriques : *Anostracospira rigaudi* et *Enterocytozpora artemiae*, des microsporidies infectant les crustacées *Artemia franciscana* et *Artemia parthenogenetica*. Nous avons effectué des passages successifs des parasites sur l'un, l'autre, ou une alternance des deux hôtes ; au bout de 10 passages, nous avons testé l'infectivité, la virulence, et la production de spores des lignées évoluées. Conformément aux études précédentes, *A. rigaudi* maintenait une fitness plus élevée sur *A. parthenogenetica*, et *E. artemiae* sur *A. franciscana*, dans chacun des traitements. Nos résultats suggèrent que l'infectivité est soumise à un trade-off faible, qui permet l'évolution du généralisme. Cependant, ce trait est contré par un trade-off fort pour la production de spores, qui finit par dominer les patrons de spécialisation. Par conséquent, l'évolution de la spécialisation dans ce système paraît être due à la combinaison de plusieurs formes de trade-off trait-spécifiques (Fig. 5).

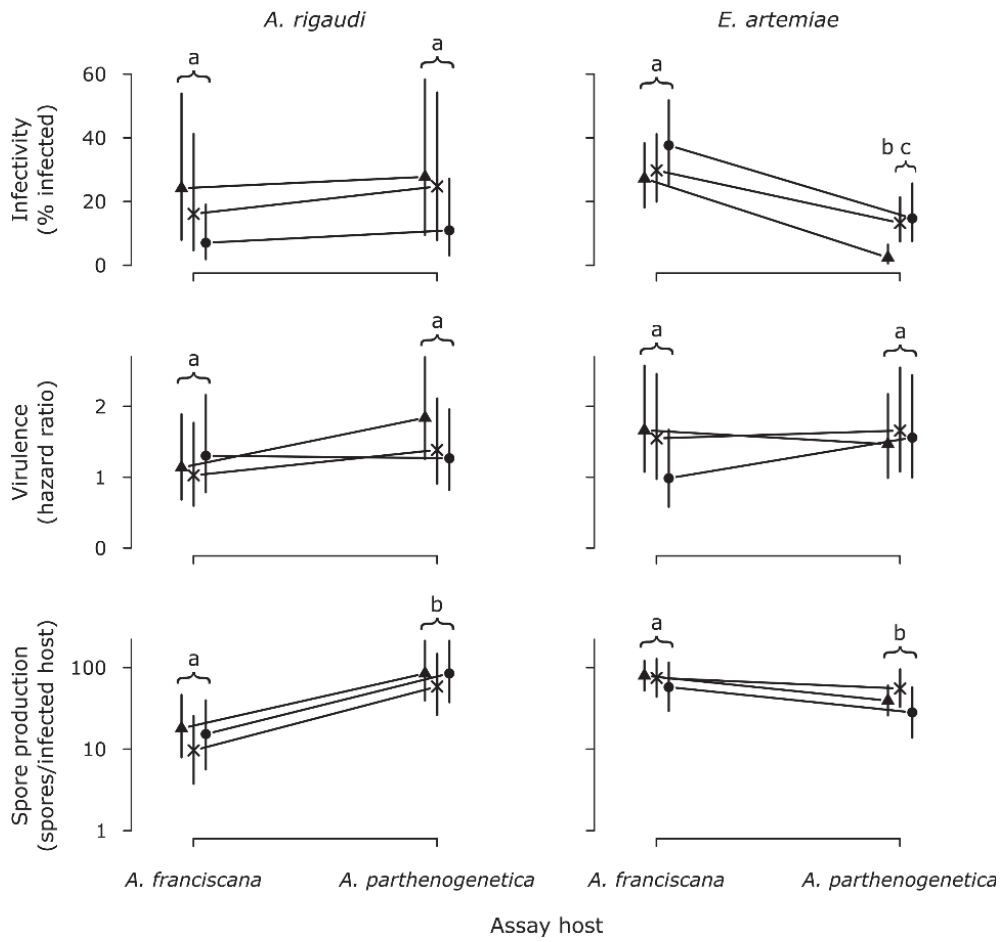


Figure 7. Infectivité, virulence, et production de spores pendant les tests finals. Infectivité est le pourcentage des hôtes infectés pendant le test court. La virulence et la production de spores ont été mesurées à l'issue du test long : la virulence est le ratio des hasards de chaque lignée comparé aux contrôles non-exposés de la même espèce, la production de spores est la quantité de spores produite par hôte (survivant et) infecté au moment du passage (échelle *ln*). Les symboles représentent le régime évolutif (triangles, 'Seul *A. f.*'; croix, 'Hôtes alternantes'; cercles, 'Seul *A. p.*') ; points noirs et lignes verticales montrent les moyennes et les IC 95%. Les différences significatives sont indiquées par des lettres ; les analyses étant faites séparément pour chaque parasite.



Artemia in Aigues-Mortes, Johan D'Espallier, 2013

Biotic challenges for extremophiles: reproductive interference and parasite specialization in Artemia

The challenges posed by biotic factors – interactions with competitors, parasites, etc. – play a large role in the evolution of populations, but are generally difficult to study. In this work, we used the *Artemia* system in the hypersaline salterns of Aigues-Mortes, France, to study the character and evolution of several interspecific interactions. The system is eminently suited to such studies: it is simple, containing two *Artemia* species, three highly prevalent parasites (one helminth and two microsporidians), and various microbiota; and many of its interactions are newly-established, because one of the *Artemia* species is invasive. We studied two types of interactions. First, the presence and evolution of reproductive interference between the native and invasive *Artemia* species. We found that the invasive *Artemia* species, which is sexual, undergoes severe interference in sex allocation when in the presence of the native, asexual *Artemia*, but no interference in mate guarding. Second, the evolution of parasite specialization in two microsporidian parasites infecting the *Artemia* species. Using field data, experimental infections, and experimental evolution, we investigated the state, causes, and consequences of specialization in the microsporidians. Results consistently indicated that the microsporidians are specialized for one host species, despite being able to complete their life cycle in either host. This specialization appears to be imposed by the precise toolkits needed to exploit each host species; in the non-specialized hosts, these triggered suboptimal exploitation, maladaptive virulence, and immunopathology. Overall, these studies suggest that the biotic factors acting on *Artemia* impose strong selection pressures, to which they cannot always respond easily. The importance of biotic factors in a system which is otherwise dominated by extreme abiotic conditions underlines the importance of species interactions in shaping evolution.

Keywords: reproductive interference, sex allocation, mate guarding, parasite specialization, parasite seasonality, virulence, trade-offs, maladaptation, interspecific interactions

Défis biotiques pour extrêmophiles :

l'interférence reproductive et la spécialisation parasitaire chez Artemia

Les défis posés par des facteurs biotiques – les interactions avec des compétiteurs, des parasites, etc. – jouent un rôle important dans l'évolution des populations, mais sont souvent difficiles à étudier. Dans cette étude, nous avons utilisé le système biologique *Artemia* dans les salins d'Aigues-Mortes, France, pour étudier le caractère et l'évolution de plusieurs interactions interspécifiques. Le système est particulièrement bien adapté à cette recherche : il est simple, contenant deux espèces d'*Artemia*, trois parasites fortement prévalents (un helminthe et deux microsporidies), et des microbiota variés ; et beaucoup des interactions du système ont été nouvellement établies, car une des espèces d'*Artemia* est invasive. Nous avons étudié deux types d'interactions. Premièrement, la présence et l'évolution de l'interférence reproductive entre les espèces native et invasive d'*Artemia*. Nous avons trouvé que l'espèce invasive, qui est sexuée, subit une interférence sévère en allocation des sexes en présence de l'espèce native et asexuée, mais pas d'interférence en gardiennage de partenaire. Deuxièmement, l'évolution de la spécialisation de deux parasites microsporidiennes qui infectent les espèces d'*Artemia*. En nous basant sur des données de terrain, des infections expérimentales, et de l'évolution expérimentale, nous avons investigué l'état, les causes, et les conséquences de la spécialisation des microsporidies. Les résultats démontrent que les microsporidies sont spécialisées dans l'utilisation d'une des espèces d'hôte, même si elles sont capables de compléter leur cycle de vie dans chacun des deux hôtes. Cette spécialisation paraît être imposée par les outils précis nécessaires à l'exploitation de chaque hôte ; dans les hôtes non-spécialistes, ils provoquaient de l'exploitation sous-optimale, de la virulence maladaptative, et de l'immunopathologie. En général, ces études suggèrent que les facteurs biotiques imposent des pressions de sélection fortes sur *Artemia*, auxquelles elles ne peuvent pas toujours répondre facilement. L'importance des facteurs biotiques dans un système qui est autrement dominé par des conditions abiotiques extrêmes souligne l'importance des interactions interspécifiques dans l'évolution.

Mots-clés: interférence reproductive, allocation des sexes, gardiennage de partenaire, spécialisation parasitaire, saisonnalité parasitaire, virulence, maladaptation, interactions interspécifiques