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LETTER

Why join groups? Lessons from parasite-manipulated *Artemia*

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Abstract

Grouping behaviours (*e.g.* schooling, shoaling and swarming) are commonly explicated through adaptive hypotheses such as protection against predation, access to mates or improved foraging. However, the hypothesis that aggregation can result from manipulation by parasites to increase their transmission has never been demonstrated. We investigated this hypothesis using natural populations of two crustacean hosts (*Artemia franciscana* and *Artemia parthenogenetica*) infected with one cestode and two microsporidian parasites. We found that swarming propensity increased in cestode-infected hosts and that red colour intensity was higher in swarming compared with non-swarming infected hosts. These effects likely result in increased cestode transmission to its final avian host. Furthermore, we found that microsporidian-infected hosts had both increased swarming propensity and surfacing behaviour. Finally, we demonstrated using experimental infections that these concurrent manipulations result in increased spore transmission to new hosts. Hence, this study suggests that parasites can play a prominent role in host grouping behaviours.

Keywords

Aggregation, *Anostracospora rigaudi*, cestode, *Enterocytospora artemiae*, *Flamingolepis liguloides*, microsporidian, swarming-surfacing manipulation.

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INTRODUCTION

Grouping is usually considered an adaptive behaviour (Alexander 1974; Ambler 2002) that provides anti-predatory (Krause & Godin 1994), reproductive (Sullivan 1981), energetic (Ritz 2000) or foraging benefits (Foster *et al.* 2001). In altruistic species, grouping with related individuals can further increase the benefit of aggregation (Young *et al.* 1994; Fraser *et al.* 2005). These benefits are supposed to outweigh grouping costs such as increased predation (Kemp 2012), reduced food availability (Spieler & Linsenmair 1999), increased energetic expenditure (Buskey 1998) or increased exposure to parasites (Côté & Poulin 1995).

Intriguingly, one non-adaptive explanation is rarely considered, namely that grouping behaviour results from parasite manipulation. Host behavioural manipulation by infecting parasites is widespread in nature, and most often evolves at the expense of host fitness (Barnard & Behnke 1990; Moore 2002; Poulin 2010). Hence, if grouping promotes transmission, parasites could manipulate their hosts into aggregating. The transmission benefits could arise differently for parasites with indirect vs. direct life cycles. For example, the former might benefit from an increased predation of groups compared with solitary individuals, whereas the latter might benefit from a greater opportunity for transmission from infected hosts within rather than outside groups.

We investigated this hypothesis in the brine shrimp *Artemia* (Anostraca). Swarming has indeed been documented for both the bisexual American species *A. franciscana* (Mason 1966) and the asexual Old World species *A. parthenogenetica* (Sánchez *et al.* 2012). In nature, swarms vary from 20 cm to 2 m in diameter (Lenz 1980); they are highly dynamic and short-lived (typically less than 1 h, Lenz 1980; authors pers. obs.). The parasite manipulation hypothesis is a good candidate to explain these swarms, as *Artemia* species host highly prevalent parasites (cestode, Georgiev *et al.* 2005; microsporidians, Ovcharenko & Wita 2005). Manipulative cestodes could increase transmission to their final avian hosts if birds prey disproportionately upon conspicuous *Artemia* swarms. Manipulative microsporidians could increase their direct transmission in dense groups if groups provide greater opportunities of spore ingestion by new *Artemia* hosts.

Furthermore, alternative hypotheses fail to fully account for swarming in *Artemia*. First, individual response to the same stimulus (e.g. light or thermal current, Mason 1966; Gulbrandsen 2001) can lead to an aggregated distribution of individuals, but it does not account for the extreme and dynamic aggregation in homogeneous habitats where swarms are typically observed. Second, swarming appears unrelated to reproduction in asexual *A. parthenogenetica* and in juvenile *A. franciscana*. Third, it is unlikely to be related to foraging or respiration: swarms do not depend on the presence of algae (Gulbrandsen 2001) and oxygen gradients are very low in natural habitats and not at the scale of swarms (Thiéry & Puente 2002). Fourth, swarms are unlikely to serve as a protection against visual predation (Gulbrandsen 1991): fish predators are absent at salinities above 100 gL^{-1} NaCl, where most *Artemia* populations are found (Britton & Johnson 1987) and swarms are observed even in the absence of bird predators (authors pers. obs.).

We investigated the parasite manipulation hypothesis in a population of *Artemia* in the Aigues-Mortes salterns (Southern France), where *A. franciscana* and *A. parthenogenetica* occur in sympatry (Sánchez *et al.* 2012) and where swarms are present from May to September (authors pers. obs). In this population, *Artemia* are infected by several parasites, but we focussed on the three most prevalent: the cestode *Flamingolepis liguloides* infects only *A. partheno-*

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genetica, the microsporian *Anostracospora rigaudi* infects mostly *A. parthenogenetica*, and another microsporidian *Enterocytospora artemiae* infects mostly *A. franciscana* (Rode N.O., Landes J., Lievens E.J.P., Flaven E., Segard A., Jabbour-Zahab R., Michalakis Y., Agnew P., Vivarès C.P. & Lenormand T. unpublished data). We examined two main hypotheses:

(1) *F. liguloides* causes swarming behaviour. *F. liguloides* has an indirect life cycle, with *A. parthenogenetica* as its intermediate and the Greater Flamingo as its final host (Georgiev *et al.* 2005). This hypothesis implies that *F. liguloides* manipulates infected *Artemia* into forming conspicuous swarms and that flamingos prey disproportionately upon these swarms. Infection by *F. liguloides* is already known to cause several phenotypic changes in *A. parthenogenetica*: colour change from transparent to red (Sánchez *et al.* 2006), castration, longer life span and higher nutritive value (Amat *et al.* 1991), and possibly surfacing behaviour (Sánchez *et al.* 2007). Under this hypothesis, we expect a higher prevalence of *F. liguloides* in swarms. Red colour and surfacing behaviour may contribute to swarm visibility. As *F. liguloides* does not infect *A. franciscana*, we expect swarms to consist only of *A. parthenogenetica*. Hence, this first hypothesis fails to explain the existence of swarms in the two American *A. franciscana* populations where *F. liguloides* is absent (Sánchez *et al.* 2012).

(2) Microsporidians contribute to swarm formation. Both *A. rigaudi* and *E. artemiae* have a direct life cycle, where spores are released with the faeces of infected individuals and then ingested by new hosts (Rode N.O., Landes J., Lievens E.J.P., Flaven E., Segard A., Jabbour-Zahab R., Michalakis Y., Agnew P., Vivarès C.P. & Lenormand T. unpublished data). These microsporidians could induce infected individuals to join swarms to access the high densities of recipient hosts. Under this hypothesis, we expect a higher prevalence of *Artemia* infected with microsporidians in swarms (with possible differences between the two microsporidian parasites or between the two *Artemia* hosts). This hypothesis accounts for the widespread occurrence of swarms across *A. franciscana* and *A. parthenogenetica* populations, but it cannot explain how parasites obtain a better transmission to uninfected *Artemia* if the latter do not join swarms.

MATERIAL AND METHODS

Sampling

To test our two hypotheses, we sampled *Artemia* spp. in five different shallow salterns (depth < 30 cm) in May 2011 in Aigues-Mortes, France. The sampling locations were chosen based on the presence of swarming *Artemia*. At each location, we sampled within and outside swarms using mesh nets with a rectangular opening of 7 × 10 cm. To avoid bias due to local heterogeneity in prevalence, we replicated sampling for two distant swarms in Site 1 and Site 2 (Table S1). Cestodes are suspected to manipulate their hosts into surfacing (Sánchez *et al.* 2007); to avoid this bias, we took depth-stratified samples wherever possible. Thus, the term 'sample' refers to *Artemia* captured in the same location, at a certain depth and either inside or outside a swarm.

Phenotypic characterisation and infection status of individuals

For each of the samples, we described the phenotype and infection status of a random subset of ~100 adult *Artemia* (min: 48; max: 227). For each individual, we first recorded the species, sex, reproductive status (reproducing or non-reproducing), body length and number of infecting *F. liguloides* cysticercoids using a binocular. Other cestodes of low-prevalence species (*Fimbriarioides tadornae*, *Eurycestus avoceti*, *Flamingolepis flamingo*, *Wardium stellorae*) were also recorded but not identified to the species level. For *A. parthenogenetica* females, we were interested in the relationship between infection by *F. liguloides*, colour change and castration, so we also measured the red colour intensity of mature *A. parthenogenetica* individuals from all samples except sample 2. To do this, individuals were anaesthetised with 50% carbonated water and photographed (Nikon D300S). We used the software ImageJ (Schneider *et al.* 2012) to quantify the relative red intensity of each individual (discounting dark areas: digestive tract, eyes, cysts). All *A. parthenogenetica* and one random third of *A. franciscana* individuals were conserved in ethanol for molecular analyses. Larger samples of *A. parthenogenetica* were taken to increase statistical power as, contrary to *A. franciscana*, *A. parthenogenetica* is infected by both cestode and microsporidians. The presence of *A. rigaudi* and *E. artemiae* was detected in each individual *Artemia* by PCR using species-specific primers of the 16S SSU (Rode N.O., Landes J., Lievens E.J.P., Flaven E., Segard A., Jabbour-Zahab R., Michalakis Y., Agnew P., Vivarès C.P. & Lenormand T. unpublished data). Because of the occurrence of so-called 'rare' males in *A. parthenogenetica*, the species of each male was confirmed using species-specific microsatellite markers (Muñoz *et al.* 2008). We found ten 'rare' *A. parthenogenetica* males in total (for 977 *A. parthenogenetica* females).

Identification of *A. parthenogenetica* genotype

Since *A. parthenogenetica* clones may be distantly related and differentially affected by parasites, we genotyped a random subset of *A. parthenogenetica* individuals (n = 187) from the two best depth-stratified sites (Site 2, replicate 2 and Site 3; mean number of individuals per sample = 19, min = 6, max = 32). Individuals that differed by one mutation (or less) at 12 microsatellite loci (Flaven *et al.* in prep) were assigned the same multilocus clonal genotypes (hereafter genotype). For statistical robustness, we only discriminated common genotypes and all genotypes with an average frequency below 8% were pooled in a single class.

Statistical analyses of *Artemia* phenotype and genotype effects

Our statistical analyses consisted of two main arcs: first, analyses of *Artemia* phenotypic traits to estimate parasite manipulation; second, analyses of swarming and infection probability including *Artemia* genotype effects. All analyses were performed using the stats package in R (R 2.14.2, http://www.r-project.org/) and model selection was based on the corrected Akaike Information Criterion (AICc; Hurvich & Tsai 1989).

We conducted a first arc of analyses with the full dataset (n = 1230) to examine the factors predicting two aspects of the *Artemia* phenotype, which we hypothesised to be under the influence of parasites: swarming behaviour and red colouration. First, we analysed the probability that a given individual was found swarming, given its sampling location and depth (hereafter the swarming probability). We used generalised linear models (GLMs) with Bernouilli error distributions. Importantly, since sampling was non-random and not proportional to *Artemia* density, the interaction between

sample and factorial distance from the surface was included in every model. Additional effects were then tested: host species, sex, length, presence of each parasite (*F. liguloides*, *A. rigaudi*, *E. artemiae*) and their double interactions. To investigate surfacing behaviours, we further included the interaction between distance (or squared distance) from the surface as a continuous covariate and all other variables. Models considering the number of *F. liguloides* cysticercoids instead of their presence/absence were also included. As a second phenotypic trait, we investigated the red colour intensity of *A. parthenogenetica* individuals using linear models. Models included sex, length, female reproductive status, swarm effect (sampled within or outside swarm), continuous distance from the surface, sample, number of *F. liguloides* cysticercoids and presence of each microsporidian parasite (*A. rigaudi* or *E. artemiae*), as well as all double and triple interactions.

In the second arc, we restricted our data set to 333 individuals (the 187 genotyped *A. parthenogenetica* and 146 *A. franciscana* from the same locations) to test for differences in swarming probability and infection status between host species/genotypes (fixed effect). For swarming probability, we used the best model from the swarming analysis as a base, from which we specifically tested for interactions between host species/genotype and parasite presence (*F. liguloides, A. rigaudi* or *E. artemiae*) and between host species/genotype and the squared distance from the surface. For the probability of infection with each parasite species, we used a GLM with a Bernoulli error distribution. We included the same effects as above, specifically investigating whether infection probability differed between host species/genotype and/or different depth. Infection by other cestodes could not be investigated (prevalence < 1.4%).

Effect of the vertical position of infected individuals on the transmission of *E. artemiae*

Prompted by depth-dependent effects in our data (see below), we hypothesised that microsporidian transmission is highest when infected hosts are alive and swimming above uninfected hosts. We tested this hypothesis using *E. artemiae* as a model; we considered *E. artemiae* to be representative of both microsporidians because of their phylogenetic proximity and because it has the weakest manipulative effects (see below). We had three treatments, each consisting of 7 *A. franciscana* and 9 *A. parthenogenetica* uninfected recipient hosts and 20 *A. franciscana* donor hosts from a natural population with high *E. artemiae* prevalence (> 80%). To differentiate infection through grazing from infection through spore ingestion in the water column, we placed recipient hosts in cylindrical cages (diameter, 10 cm; height, 10 cm) at the bottom of a tank that allowed (Treatment A, Fig. 5a) or prevented (Treatment B, Fig. 5b) grazing of detritus and spores, while donor hosts were placed in a cage above them (Fig. 5a, b). In treatment A and B, dead donor hosts were removed daily so that spores came only from live hosts. In a third treatment, uninfected recipient hosts were placed in the upper cage, whereas donor hosts were placed in the lower cage and not allowed to graze (Treatment C, Fig. 5c). Cages were separated by a net (a similar net was used to prevent grazing in treatments A and C, mesh size 0.2 mm). To compare spore transmission from living and dead hosts, we placed 16 uninfected recipient individuals in a tank with a homogenate of 20 donor hosts (positive control D, Fig. 5d). Finally, we kept 16 uninfected individuals isolated in a tank (negative control E, Fig. 5e). We replicated each treatment four times and each control twice. All recipient hosts were PCR-tested for infection after 10 days of exposure.

For the statistical analysis, we used a GLM with a Bernoulli error distribution. We included host recipient species and sex in all models. We then compared models including treatment effect, replicates and their interaction. To specifically test for differences in transmission from living vs. dead hosts, we also included a model in the comparison where infection probability was constrained to be identical in treatment A, B and D.

RESULTS

Effect of parasite infection on *Artemia* spp. phenotype

Parasite and depth effects were the strongest factors influencing the swarming probability of both *A. parthenogenetica* and *A. franciscana*. All best models included the presence of the three parasites (ΔAICc > 2 for any model excluding *F. liguloides*, *A. rigaudi* or *E. artemiae* effects, Table S2); individuals infected by any of these parasites were more likely to be swarming (Fig. 1). In contrast, support for an effect on swarming of other cestode species was very low (ΔAICc = 1.70, Table S2). *A. rigaudi* and *E. artemiae* had a synergistic effect on the swarming behaviour of co-infected hosts (Table S2). Importantly, a negative interaction between the presence of each microsporidian and the square distance from the surface was found in all best models, indicating that individuals infected by *A. rigaudi* or *E. artemiae* were more likely to be found swarming when near the surface (Fig. 1). Swarming was independent of depth in *F. liguloides*-infected individuals (Fig. 1). *A. parthenogenetica* tended to swarm slightly more than *A. franciscana* (ΔAICc = 1.07, Table S2). The swarming propensity of large individuals varied across samples (Table S2). Surprisingly,

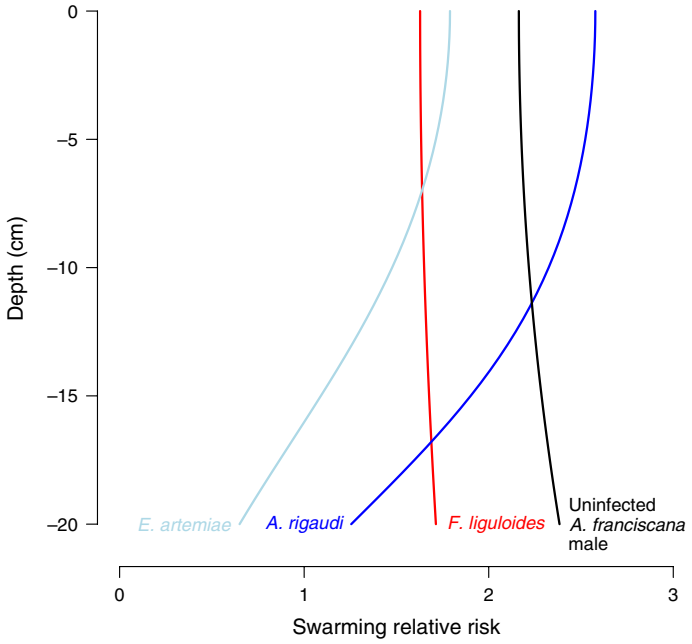

Figure 1 Effect of parasite infection and sex on *Artemia* spp. swarming behaviour. Swarming relative risk represents the swarming probability of a focal individual (infected female or uninfected *A. franciscana* male) divided by the swarming probability of an uninfected *A. franciscana* or *A. parthenogenetica* female (there was no strong difference in female swarming behaviour between both species).

males were more likely to swarm than females (Fig. 1, Table S2). However, as only 10 *A. parthenogenetica* 'rare' males were sampled, we had low power to investigate the species-dependence of this effect. Finally, there was a negative interaction between sex and *A. rigaudi* infection, indicating that *A. rigaudi*-infected males were less likely to swarm than expected from additive effects (Table S2).

Analysis of *A. parthenogenetica* red colouration indicated a positive covariation between swarming behaviour and red colour intensity in infected individuals. The best models included a positive interaction between swarming effect and *F. liguloides* cysticercoid count, indicating that swarming infected individuals were redder than non-swarming infected individuals and that this colour alteration increased with the number of infecting cestode larvae (Fig. 2, Table S3). Among non-swarming individuals, infected *Artemia* also tended to be slightly redder (ΔAICc = 0.68, Table S3). Independently of this swarming effect, we found an augmented red colour in non-reproducing females (Fig. 2, Table S3). As 96% of these females were infected with *F. liguloides*, which castrates its hosts (Amat *et al.* 1991), the higher red intensity of non-reproducing females is likely an indirect effect of cestode castration and of the subsequent accumulation of carotenoids and/or haemoglobins (for which different types are present in *Artemia*, Gilchrist & Green 1960; Bowen *et al.* 1969). Infection with microsporidians or other cestode species did not alter the effect of *F. liguloides* on red colour intensity (ΔAICc > 1.8, Table S3). Finally, red colour intensity increased with length (ΔAICc > 2, Table S3), which suggests an accumulation of carotenoids/haemoglobins with age.

Effect of host species and genotype on swarming and infection probabilities

When *A. parthenogenetica* genotypes were taken into account, we found that host genotype impacted several of the factors predicting swarming probability. The host species/genotype factor included the 4 most common *A. parthenogenetica* genotypes (A–D) and two additional levels pooling the least common genotypes (E) and *A. franciscana* individuals (*Af*). Support for a difference in swarming behaviour between *A. franciscana* and most *A. parthenogenetica* genotypes (B, C, E) was again low (ΔAICc = 0.95, Table S4). Genotypes A and D had a higher *F. liguloides*-induced swarming propensity than the other genotypes (ΔAICc < 2, Table S4). Surprisingly, all best models included an interaction between the genotype factor and the distance from the surface (ΔAICc < 2, Table S4), indicating that depth-dependent swarming propensity differed across genotypes. Support for the synergistic effect of *A. rigaudi* and *E. artemiae* coinfection was much lower when taking host genotype into account (ΔAICc = 2.1, Table S4). Similarly, the interaction between length and sample previously found was not supported (ΔAICc = 4.4, Table S4). A likely explanation is that length varied across genotypes in the different samples, leading to this spurious effect in the global analysis.

Parasite infection probability differed across species but not across genotypes for *F. liguloides* (ΔAICc > 2, Fig. 3, Table S5). In contrast, the probability that an individual was infected by a microsporidian parasite was species- and genotype-dependent. *A. rigaudi* prevalence was lowest in *A. franciscana* individuals and *A. parthenogenetica* genotype D (ΔAICc < 2) with a similar trend in genotype A (ΔAICc = 1.1, Fig. 3, Table S6), whereas *E. artemiae* prevalence was highest in *A. franciscana* and these two genotypes (ΔAICc < 2, Fig. 3, Table S7). Parasite prevalence was highly consistent across sampling sites, except for *A. rigaudi* in genotype A (Fig. 3).

Modelling the infection probability also allowed us to investigate the depth-dependent prevalence of parasites outside and inside the swarm. Surprisingly, *A. rigaudi* and *E. artemiae* infection increased with the distance from the surface outside swarms, indicating that

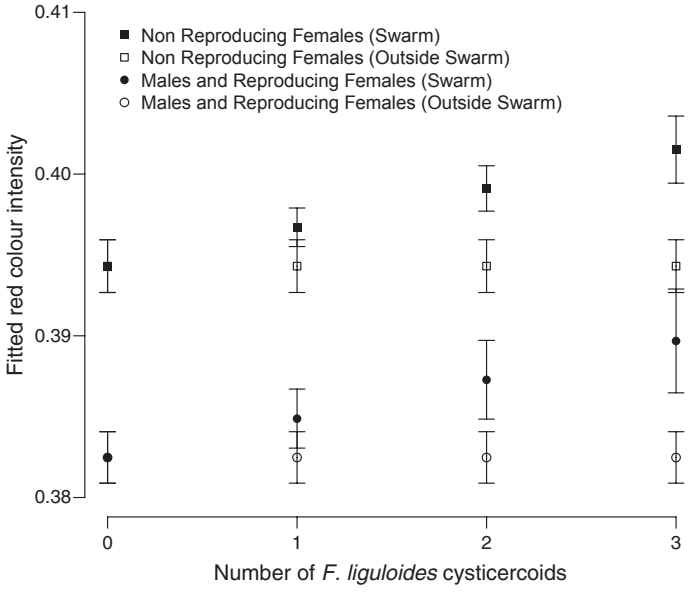

Figure 2 Covariation between number of *F. liguloides* and red colour. Estimates correspond to the red colour intensity of individuals sampled outside the swarm (open symbols) or within the swarm (closed symbols). Error bars represent estimates' 95% confidence intervals. (R^2 = 0.34).

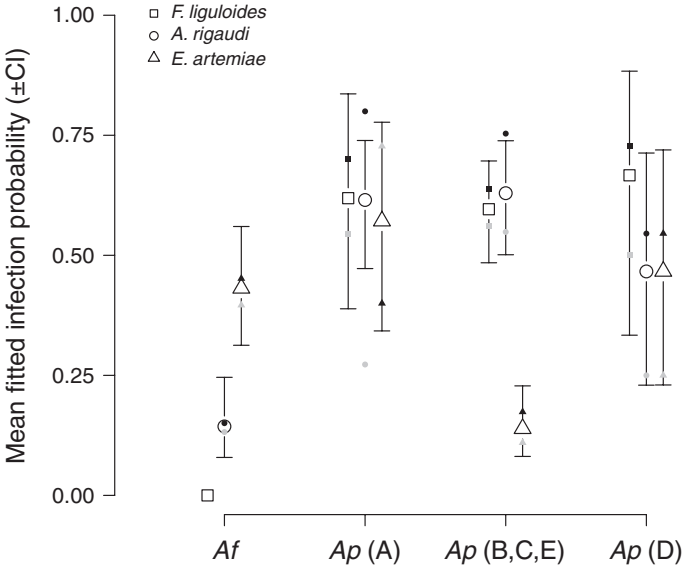

Figure 3 Infection probabilities of *A. franciscana* and the different *A. parthenogenetica* genotypes. For each of the three parasites, fitted infection probabilities (open symbols) were computed for each individual in the dataset using the corresponding best model. Observed infection probabilities were calculated for samples 4 (grey symbols) and 5 (black symbols). No *F. liguloides* infection was found in *A. franciscana*. Error bars represent estimates' 95% confidence intervals.

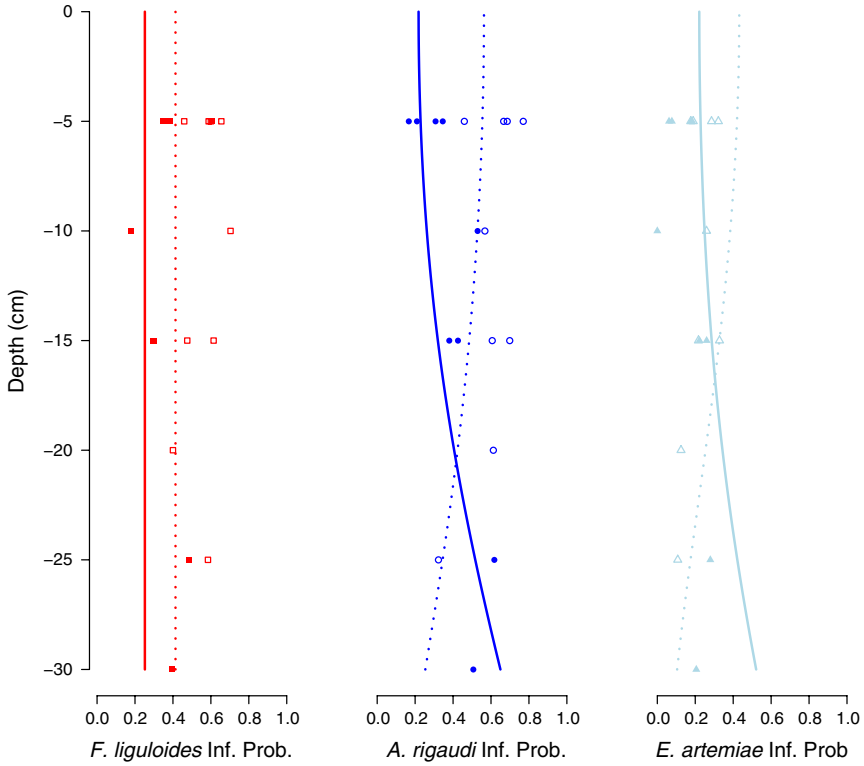

Figure 4 Fitted and observed parasite infection probability (Inf. Prob.) of each of the three parasites as a function of depth. Fitted estimates for individuals outside (solid lines) and inside (broken lines) swarms were calculated with the same model as Fig. 3, by taking into account the average proportion of the different species/genotypes found in samples 4 and 5. For comparison, the observed infection probabilities (samples 1–5) are given for individuals sampled outside (filled symbols) and inside (open symbols) swarms.

infected individuals were more likely to be found at the bottom outside swarms (Fig. 4, Table S6–S7). A contrasting trend was apparent for *F. liguloides*, with low support (ΔAICc = 0.6, Table S5). Again, parasite prevalence was consistently higher within swarms, with a strong increase towards the surface for microsporidian parasites (Fig. 4).

Effect of the vertical position of infected individuals on the transmission of *E. artemiae*

The vertical position of donor hosts had clear effects on the experimental transmission of microsporidians, as did their life status (Fig. 5). Observed *E. artemiae* infection was the highest when uninfected recipients hosts were placed at the bottom of the tank and allowed to graze detritus (92%, treatment A). Infection lowered when individuals were prevented from grazing (81%, treatment B) or fed crushed donor hosts (70%, control D). Infection was the lowest when uninfected hosts were placed above donor hosts (22%, treatment C). None of the hosts in negative control E became infected. The best model fitted a different effect to each treatment (Table S8, Fig. 5); the model specifying the same transmission probability from living and dead hosts fitted the data poorly (ΔAICc > 2, Table S8). Hence, *E. artemiae* transmission occurs optimally from live hosts (most likely through the faeces as in related *Daphnia* gut microsporidians, Ebert *et al.* 2000) and spore transmission was highest when donor hosts were alive and swimming above recipient hosts.

DISCUSSION

Parasite manipulation of swarming behaviour

Remarkably, our results strongly suggest that *F. liguloides*, *A. rigaudi* and *E. artemiae* manipulate *Artemia* swarming behaviour: all three parasites were consistently much more prevalent in swarms. Before interpreting our results in the light of the 'parasite manipulation hypothesis', we consider different alternative explanations. First, the observed pattern cannot be a consequence of recent transmission occurring in swarms, which are short-lived (< 1 h), or during transportation after sampling. *F. liguloides* cysticercoids are indirectly transmitted through flamingos, so that there is no reason to expect a higher *F. liguloides* prevalence due to within-swarm transmission. *A. rigaudi* and *E. artemiae* infections cannot be detected by PCR in the first two days after infection (Rode N.O., Landes J., Lievens E.J.P., Flaven E., Segard A., Jabbour-Zahab R., Michalakis Y., Agnew P., Vivarès C.P. & Lenormand T. unpublished data), a period which is much longer than the duration of the swarms. Thus, it is unlikely that the higher prevalence of microsporidians in sampled swarms resulted from direct transmission occurring in those swarms. We can also exclude the possibility that these results occur because of swarming restricted to older, more parasitised, individuals as our models account for individual size (which is highly correlated to age, Medina *et al.* 2007). Similarly, swarming is not restricted to some species/genotypes that happen to be more susceptible to infection:

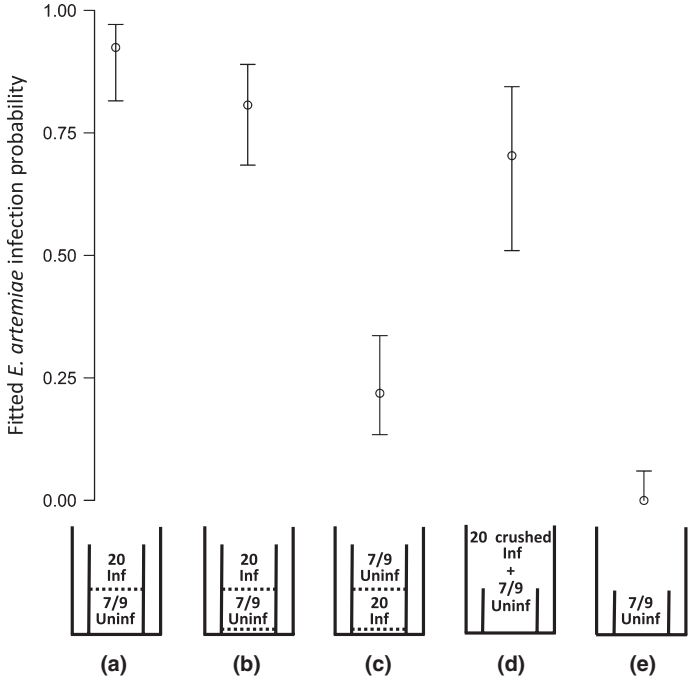

Figure 5 Fitted *E. artemiae* infection probabilities. (a–b) 7 *A. parthenogenetica* and 9 *A. franciscana* recipient hosts were placed below infected hosts [with grazing at the bottom allowed (a) or prevented (b)]. (c) Recipient hosts were placed above infected hosts (grazing prevented). (d) Recipient hosts were fed crushed infected hosts. (e) Uninfected hosts were kept isolated (negative control). Four (a–c) or two (d–e) replicates per treatment. Error bars represent estimates' 95% confidence intervals.

the increased parasite infection in swarms holds true after controlling for differences between species/genotypes. Finally, swarming is unlikely to be an adaptive host response to parasite infection, such as a compensatory mechanism with regards to resource acquisition. Indeed, the large density increase in swarms can only have a negative impact on the availability of resources (algae, oxygen) within them. Hence, although we cannot completely dismiss alternative hypotheses, these appear very unlikely.

By causing an aggregation of intermediate hosts that can easily be preyed upon by a filter-feeder, *F. liguloides* is likely to increase *A. parthenogenetica* predation by, and hence transmission to, its final host, the Greater Flamingo. Although the predation rates of swarms have not yet been studied, we find that *F. liguloides* manipulates host colouration in a way which fits with this hypothesis. *F. liguloides* is known to castrate its host, which is thought to increase survival and red pigmentation (Amat *et al.* 1991), and indeed we find that *F. liguloides* castrates its host and that castration increases red colouration (Fig. 2). However, *F. liguloides* also manipulates host colour directly: in swarms, *F. liguloides*-infected individuals are redder than expected after controlling for castration. This may further increase the visibility of the swarms for the flamingos. We did not find any depth effect of cestode infection (Fig. 4), which is congruent with the observation that flamingos can filter-feed at different depths, depending on the availability of resources (Jenkin 1957). Importantly, flamingos might have difficulties evolving a response to avoid parasitised swarms, as this would require a decreased feeding rate which might be counter-adaptive (Kuris 2003).

By manipulating hosts into joining dense swarms, microsporidians may enjoy increased direct transmission to new hosts. Consistent with this hypothesis, we found that the swarming probability was highest close to the surface (Figs 1 and 4), a position which strongly increases transmission to uninfected hosts swimming below in the water column, while reducing the chance of infection from above (Fig. 5b). We demonstrated this depth effect experimentally using *E. artemiae*. This parasite is phylogenetically close to *A. rigaudi* and other microsporidian gut parasites (Rode N.O., Landes J., Lievens E.J.P., Flaven E., Segard A., Jabbour-Zahab R., Michalakis Y., Agnew P., Vivarès C.P. & Lenormand T. unpublished data), which have been shown to induce surfacing behaviour in their cladoceran hosts (Fels *et al.* 2004; Fels 2006). However, our experiment is the first to demonstrate that surfacing is associated with a transmission advantage.

Intriguingly, swarming must have already been present as a host behaviour before the evolution of these microsporidian manipulations. In the bisexual *A. franciscana* species, males were twice as likely to swarm as females (Fig 1.), which suggests that males actively enter swarms, and thus that swarming might be important for reproduction. This is however obviously not the case for the asexual *A. parthenogenetica*. Thus, we hypothesise that swarming first evolved as a mating behaviour in sexual *Artemia* such as *A. franciscana*. This hypothesis implicitly supposes significant reproductive benefits for swarming *A. franciscana*. Experimental tests could help determine whether parasite-free *Artemia* (especially females) enter swarms actively or passively. Asexual *A. parthenogenetica* were recently derived from sexual species (Baxevanis *et al.* 2006) and we hypothesise that their swarming behaviour could be a residual version of the reproductive behaviour of their sexual ancestor. Alternatively, swarming in *A. parthenogenetica* may have first occurred as a consequence of *F. liguloides* manipulation. Microsporidian parasites could have taken advantage of these behaviours to infect new hosts and would have evolved surface-swarming manipulation secondarily.

Trade-off associated with swarming manipulation

Swarming is likely to increase the transmission success of each parasite, but the resultant spatial aggregation of infected hosts can be expected to impose costs on manipulation as well. These costs depend both of the frequency and the density of infected hosts. Each parasite must balance the benefit of swarming (an increased rate of transmission) with the costs (a higher risk of coinfection and unsuitable predation). For each parasite, we expect different factors to affect the outcome of this trade-off. For *F. liguloides*, an increased prevalence of conspecific parasites inside swarms might translate into an increased flamingo predation (highly parasitised swarms being the reddest) but also into an increased competition for resources inside the final host. Furthermore, the relative predation rates of swarming *Artemia* by flamingos and non-host predators will determine the costs and benefits of swarm induction by cestodes (Fig. 6a). For pecking birds such as waders, catching individual *Artemia* may be difficult at high *Artemia* densities (Verkuil *et al.* 2003). However, swarming may increase predation by non-host filter-feeders (i.e. shelducks), which remains to be investigated. Similarly, an increased transmission of *A. rigaudi* and *E. artemiae* spores is expected to trade-off against the risk of coinfection and predation of their *Artemia* hosts (Fig. 6b). Microsporidian spores are more likely to be ingested by new hosts when the infected host is swarm-

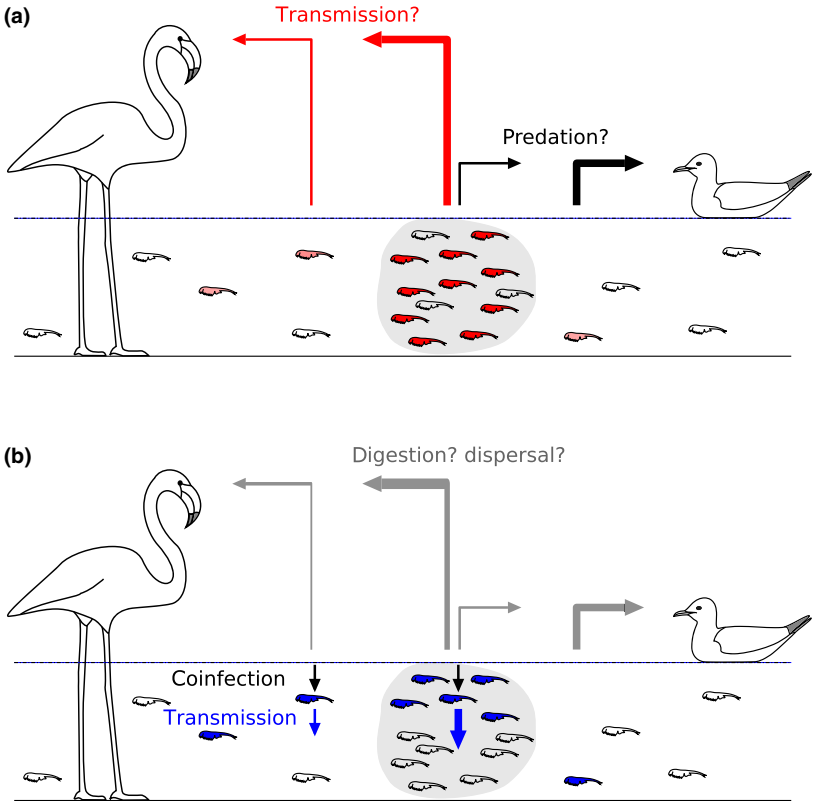

Figure 6 Trade-off between cestode (a) and microsporidian (b) parasite transmission and predation/coinfection. Upward left and right arrows represent the respective advantages and costs of manipulation for parasites. (a) *F. liguloides* has a higher prevalence within than outside swarms (proportion of shaded vs. unshaded hosts). *F. liguloides* transmission to the final host (flamingo, left) might be increased when infecting swarming (dark shaded) compared with non-swarming (light shaded) hosts. Swarming might also decrease the risk of predation by pecking birds (seagull, right). (b) *A. rigaudi* and *E. artemiae*-infected *Artemia* (shaded hosts) are preferentially found in swarming-surfacing *Artemia*, which results in an increased spore transmission to hosts swarming underneath, while coinfection risk is limited (downward arrows). Avian predation might either be advantageous or deleterious.

ing (Fig. 6b). However, the increased prevalence of microsporidians inside swarms is also likely to be costly: swarm-inducing microsporidians risk multiple infection of their host, and the establishment of their spores in new hosts could be seriously impaired by inter- and intraspecific competition. The balance of this transmission/coinfection trade-off will depend on the density of individuals, the intensity of surfacing manipulation, and the proportion of uninfected individuals within and outside the swarm, as well as the effect of coinfection on establishment and persistence. Besides the risk of coinfection, an increase in levels of avian predation due to swarming may be costly for microsporidian parasites (Fig. 6b), especially given the induction of surfacing behaviour. However, it remains unknown how well microsporidian spores survive bird digestive systems: the effect of predation may range from disastrous (fully digested) to beneficial (dispersed into other salterns).

Host genotype influences parasite fitness

Parasite fitness depends on successful infection and manipulation, which may vary with host genotype. For *F. liguloides*, *A. franciscana* hosts were entirely free from infection. As *F. liguloides* can infect the more distantly related *A. salina* (Amarouayache *et al.* 2009), these results suggests that host genetics play an important role in *F. liguloides* infection. Within *A. parthenogenetica*, host genotype did not affect the cestode's ability to infect its hosts, but genotypes A and D were more subject to behavioural manipulation by this parasite. These results provide further evidence that host genetic variation plays a major role in parasite manipulation success (Martinez *et al.* 2012).

In contrast to the results for *F. liguloides*, we found that host species and genotype (for *A. parthenogenetica*) affected the susceptibility to infection but not manipulation by *A. rigaudi* and *E. artemiae*. *A. franciscana* and *A. parthenogenetica* genotypes A and D were generally more resistant to infection by *A. rigaudi* and more susceptible to *E. artemiae*. Once infected, host species/genotype did not affect the magnitude of microsporidian manipulation. These results indicate that the two microsporidian species are specialists for different host genotypes. Importantly, genetic variation for host positioning behaviour as demonstrated by our and other studies (e.g. de Meester 1993) is also likely to influence parasite fitness. Future assessment of the influence of parasite genetic variation on host infection and manipulation would shed light on the potential for host–parasite coevolution in this system.

Since the susceptibility to infection and the degree of manipulation vary among genotypes, genetically related individuals will be found aggregated in swarms. In any system with such parasite manipulation, grouping could spuriously be interpreted as a result of some form of cooperation. Hence, disentangling the respective role of parasite manipulation vs. kin selection in the aggregation of related individuals appears crucial.

Parasite manipulation of host swarming behaviour is likely to be widespread

Our results strongly suggest that parasite manipulation is a major, mostly non-adaptive, cause of *Artemia* swarming. Although the role of parasites in swarming behaviour has been invoked in fish (Ward *et al.* 2002, 2005) where alternative hypotheses are difficult to exclude, our study indicates that such manipulation may be much more widespread. First, we showed that all investigated parasites had an influence on swarming behaviour, although cestodes and microsporidians are phylogenetically very distant and have dissimilar modes of transmission. Furthermore, each microsporidian species was able to induce swarming in both *A. franciscana* and *A. parthenogenetica* hosts, which diverged ~32 Myr ago (Baxevanis *et al.* 2006). Moreover, the existence of behavioural manipulation by contact-transmitted parasites is still a matter of debate (Poulin 2010). This study demonstrates that gut microsporidians can increase their direct transmission through behavioural manipulation. Further experimental studies would help find the proximate mechanisms used for behavioural manipulation and reinforce these conclusions.

CONCLUSION

Our results strongly support the 'parasite manipulation hypothesis' and suggest that three parasites of *Artemia* manipulate their host into swarming to increase their own transmission. None of the alternative hypotheses considered could account for the pattern observed. Hence, it seems that the microsporidians *A. rigaudi* and *E. artemiae* manipulate both *A. parthenogenetica* and *A. franciscana*, while *F. liguloides* infects and manipulates *A. parthenogenetica*. Our transmission experiment demonstrated that the surface-swarming behaviour of microsporidian-infected individuals increases parasite transmission, while reducing the likelihood of coinfection. To the best of our knowledge, this is the first conclusive evidence that concurrent induction of surfacing and swarming behaviour can result in increased parasite transmission. Given the swarming behaviour of *F. liguloides*-infected and male hosts, we suggest that microsporidians induce swarming in their hosts to profit from the pre-existent swarming behaviour of cestode-infected and/or mate searching *Artemia*. The benefits of behavioural manipulation are likely to trade-off with increased risks of coinfection and predation of parasite-manipulated hosts. Finally, we find that genetic variation in *A. parthenogenetica* influenced both infection (microsporidian parasites) and manipulation (cestode parasite). On the basis of our results, we suggest that swarming due to parasite manipulation may be more prevalent than previously assumed.

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AUTHORSHIP

MIS, TL and NOR designed the experiment. EF, EJPL, RJZ, TL and NOR sampled the individuals. EJPL and NOR did the measurements. EF, AS, RJZ and EJPL did the molecular work. AS performed the infection experiment. EJPL and NOR analysed the data. EJPL, TL and NOR wrote the manuscript.

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