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E-Article

Infectious Dose Affects the Outcome of the Within-Host Competition between Parasites

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abstract: The ecological and epidemiological processes underlying the success of parasites competing within individual hosts are not yet clear. We investigated one idea: that increasing one parasite's infectious dose might decrease the success of its competitor. We reared uninfected larvae of the mosquito *Aedes aegypti* and exposed them to two concentrations of the microsporidium *Vavraia culicis* and of the protozoan *Ascogregarina culicis*. The rate at which *Vavraia* produced its infectious spores depended on its dose and that of its competitor: when the dose of *Vavraia* was high, only the higher dose of *Ascogregarina* slowed the production of spores, but when the dose was low, either dose of *Ascogregarina* did. *Ascogregarina* was least likely to produce oocysts when its competitor's dose was high and the mosquito was reared with little food. This was due to the mosquito's preadult mortality induced by *Vavraia*. Our results show that increasing the dose of a parasite can increase its deleterious effects on a coinfecting parasite. Since dose increases with a parasite's prevalence, such dose effects could lead to an epidemiological feedback that ultimately eliminates one of the parasites.

Keywords: coinfection, infectious dose, epidemiology, coexistence, competition.

Introduction

The simultaneous infection of the same host by several parasites can affect their transmission and, consequently, their epidemiology. Such interactions have been observed for several parasites, including some important infectious diseases of humans. In some cases, coinfection by a parasite enhances the transmission of its competitor. The malaria parasite *Plasmodium yoelii*, for example, has greater transmission from a mouse to a mosquito when the mouse is also infected by the helminth *Echinostoma caproni* than when it harbors no helminth (Noland et al. 2007). In other cases, the presence of a second, coinfecting parasite can reduce a parasite's transmission. Thus, a virulent strain of the trematode parasite *Schistosoma mansoni*, which infects the snail *Biomphalaria glabrata*, has a lower reproductive success when it competes with a strain of low virulence than when it is alone in the host (Gower and Webster 2005). Finally, the outcome of coinfection sometimes depends on the environmental conditions. In mixed infections of the herbivorous insect *Panolis flammea* by two strains of nuclear polyhedrosis virus, one of the two strains performs better in mixed infections than in a single infection if the host is fed *Pinus sylvestris*, but there is no effect of coinfection if it is fed *Pinus contorta* (Hodgson et al. 2004). Infectious dose, the number of the parasite's infectious forms to which a host is exposed, can also influence the outcome of coinfections. In fruit flies, the presence of a second parasite, *Howardula aoronymphium*, increases the reproduction of the nematode *Parasitylenchus nearticus* but only if the infectious doses of both parasites are high (Perlman and Jaenike 2001).

The effects of infectious dose on coinfections could influence the parasites' epidemiology if transmission goes on to influence future infectious dose. It would indeed be surprising if there were no such feedback between infectious dose and transmission. A parasite's infectious dose is determined by factors such as its prevalence, the rate at which it produces its infectious forms, the duration of its infection, and its mode of transmission. Each of these traits can, in turn, be influenced by infectious dose (at least in single infections). For example, the probability that the bacterium *Pasteuria ramosa* infects the freshwater crustacean *Daphnia magna* (Regoes et al. 2003; Ben-Ami et al. 2008); the rate at which the protozoan *Ophryocystis elektroscirrha* produces its infectious forms in its host, the butterfly *Danaus plexippus* (de Roode et al. 2006); and the ratio of horizontal to vertical transmission of the microsporidium *Edhazardia aedis*, which infects the mosquito *Aedes aegypti* (Agnew and Koella 1999), increase with their infectious doses. If the dose of a parasite affects not only its own transmission but also that of a competing parasite in coinfections, as suggested by the study on nematodes mentioned above (Perlman and Jaenike 2001), the epi-

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demiology of the first parasite would influence the epidemiology of the second one. Dose effects would therefore couple the population dynamics of parasites that share the same host.

We experimentally investigated the role of infectious dose for coinfections by parasites that have detrimental effects on each other. In particular, we investigated whether the detrimental effect of each parasite on its competitor increases with its infectious dose, that is, whether increasing the dose of a given parasite decreases the transmission of its coinfecting competitor. We used two parasites of the mosquito *A. aegypti*, the apicomplexan *Ascogregarina culicis* and the microsporidium *Vavraia culicis*. We furthermore varied the amount of food available to the host, since we expected that the competing parasites are more likely to suffer from competition when resources are limiting.

Material and Methods

Biological System

The yellow fever mosquito *Aedes aegypti* is widespread in many tropical and subtropical areas. As the vector of several human viruses, such as dengue and chikungunya, it is intensively studied. The larvae develop in small bodies of water, where they feed on bacteria. After at least 6 days, they transform into pupae and emerge 2 days later as adults (Christophers 1960).

The microsporidium *Vavraia culicis* is an obligate, intracellular parasite of several mosquito species (World Health Organization 1980; Becnel et al. 2005; Andreadis 2007). The host larvae become infected when they ingest the spores of the parasite along with their food. Spores appear 4–10 days after infection. In *A. aegypti*, infections have two possible outcomes (Michalakis et al. 2008). With little food or a high dose of spores, the infected larva and pupa die because of the detrimental effect of the pathogen. The death of the host enables the parasite's spores to be released into the aquatic environment and to initiate new infections. If the larvae are well fed and few spores are ingested, the larvae survive and infected adults emerge. These are smaller and survive less long than uninfected adults (Bedhomme et al. 2004). The main mode of transmission of *Vavraia* is by the death of larvae and pupae in the aquatic environment (Michalakis et al. 2008). However, the occasional release of spores in new breeding sites, probably by the death of infected, ovipositing females, is likely to occur.

The protozoan *Ascogregarina culicis* is an obligate, extracellular parasite of *A. aegypti* (Sulaiman 1992; Reyes-Villanueva et al. 2003). Host larvae are infected when they ingest the parasite's oocysts (its infectious stage) along with their food. The parasite goes through two developmental stages before producing infectious oocysts again. If infection occurs in the first days of larval life, all oocysts are produced approximately 24 h before the host's emergence (Roychoudhury and Kobayashi 2006). If infection occurs later, the oocysts are formed after emergence. The parasite has two modes of transmission. First, local transmission occurs when oocysts are released as adults emerge and when mosquitoes harboring oocysts (i.e., pupae or emerging adult) die within their breeding site. Second, distant transmission occurs when infected females shed oocysts with their eggs while ovipositing and possibly when infected adults die on water containing host larvae. Note that if infection occurs late, only distant transmission can occur.

There is therefore a conflict between the transmission of *Vavraia* and that of *Ascogregarina*. The death of larvae and early pupae is necessary for the main transmission of *Vavraia* but generally prevents the transmission of *Ascogregarina*, since it has not yet produced its oocysts.

J. J. Becnel (U.S. Department of Agriculture) provided the mosquito population and the strain of *Vavraia* he isolated from a Floridian population of *Aedes albopictus* mosquitoes. *Ascogregarina* was obtained from a natural population of *A. aegypti* mosquitoes by D. Wesson (Tulane University) in 2003 and maintained in our lab for 3 years.

Experimental Design

In a full-factorial design, we used three treatments for each parasite (uninfected controls and two doses of infection) and two food levels. Hence, there were 18 different treatments. For each of them, we reared 18 larvae individually.

On the first day of the experiment, we synchronously hatched under low pressure several hundred mosquito eggs. The following day, each larva was placed into a well of 12-well tissue culture plates with 4 mL of deionized water. We infected 2-day-old larvae by adding to their wells 0, 500, or 5,000 *Ascogregarina* oocysts (treatments referred to as no *Ascogregarina*, low Ascogregarina, and high *Ascogregarina*) and 0, 1,000, or 10,000 *Vavraia* spores (treatments referred to as no *Vavraia*, low *Vavraia*, and high *Vavraia*). These doses fall in the range of those usually employed to maintain the parasite stocks and used in studies with *Ascogregarina*, *Vavraia*, or closely related species (Kelly et al. 1981; Sulaiman 1992; Bedhomme et al. 2004; Tseng 2006; Rivero et al. 2007). They ensure >90% prevalence in single infections (S. Fellous, personal observation). Unfortunately, no information exists on the doses experienced by our parasites in their natural environment. The larvae were fed ad lib. during the first 24 h of their lives. Those reared in the high-food treatment received 0.04, 0.08, 0.16, 0.32, 0.64, and 0.32 mg of fish food (Tetramin) on the second, third, fourth, fifth, sixth, and every

following day, respectively, and those reared with low food received half of these amounts. For uninfected mosquitoes, the high-food treatment corresponds to an optimal environment and the low food to an environment in which their development is possible but slow.

After pupation, individuals were transferred with 0.15 mL of water to 1.5-mL centrifuge tubes covered with cotton wool. On the day of emergence, they were separated from the water where they emerged. Both samples were then frozen at −20°C. Dead larvae and pupae were collected daily and frozen at −20°C. The oocysts and spores found in the hosts and in the water where the adults emerged were counted with a hemocytometer and a phase-contrast light microscope (× 400).

The experiment was performed in a controlled-temperature chamber at 26° ± 2°C, 60% ± 10% humidity, and 12 h of light per day.

Statistical Analysis

For *V. culicis*, we analyzed three traits, the proportion of hosts harboring spores, the proportion of hosts that contained spores and died in the aquatic environment (i.e., the success of *Vavraia* at transmitting within the larval site through host death before adulthood), and the number of spores in those mosquitoes that contained spores. For *A. culicis*, we analyzed the proportion of hosts with oocysts, the number of oocysts (in positive ones), and the proportion of oocysts that remained in the host's breeding site (i.e., local transmission). The effects of coinfection on the host's life history will be reported in a separate article. Here, we analyzed the mortality of the host before adulthood, since this trait is important for the parasites' transmission.

We used linear models for continuous traits (i.e., number of *Vavraia* spores, number of *Ascogregarina* oocysts, proportion of locally transmitted oocysts). The numbers of *Vavraia* spores and *Ascogregarina* oocysts were log transformed in order to satisfy the assumptions of the linear model. We carried out the analyses of categorical variables (i.e., presence of *Vavraia* spores or of *Ascogregarina* oocysts, the transmission of *Vavraia* to the larval site through host death, and the host's survival until adulthood) with generalized linear models with binomial errors and controlled for overdispersion. We included in the models the doses of each parasite as ordinate factors and the food level as nominal. Because the spores of *Vavraia* appear at least 4 days after infection and their number increases with the duration of the infection, we included the time between infection and host death in the models of spore presence and spore number. However, because the durations of infection in the two food treatments overlapped only slightly, the potential interactions between these two factors could not be interpreted.

If *Ascogregarina* infects first instar larvae, as was the case in our experiment, all oocysts of the parasite are produced shortly before the host emerges (Roychoudhury and Kobayashi 2006). It was thus not necessary to correct the analyses of oocyst presence, oocyst number, and mode of transmission for the duration of the infection.

Table 1: Results of the analyses for the presence of *Vavraia culicis* spores, whether they were released in the larval environment through host death, and their number

Trait and factors	df	SS	χ^2	*F*	*P*
Spore presence:					
Ascogregarina	2		7.98		.0185
Duration of the infection	1		107		<.0001
Transmission by death of host before adulthood:					
Vavraia	2		49.8		<.0001
Food	1		55.7		<.0001
Spore number:					
Ascogregarina	2	3.30		1.35	.2631
Vavraia	1	7.15		5.83	.0170
Food	1	8.34		6.80	.0100
Duration of infection	1	22.4		18.2	<.0001
Ascogregarina × *Vavraia*	2	3.08		1.26	.2879
Ascogregarina × food	2	14.6		5.94	.0033
Ascogregarina × duration of infection	2	13.3		5.40	.0054
Vavraia × food	1	1.80		1.47	.2274
Vavraia × duration of infection	1	1.66		1.36	.2459
Ascogregarina × *Vavraia* × food	2	8.83		3.60	.0297
Ascogregarina × *Vavraia* × duration of infection	2	10.9		4.45	.0133
Error	151	185			

Note: Final models after backward elimination of insignificant terms ($P > .1$) are presented.

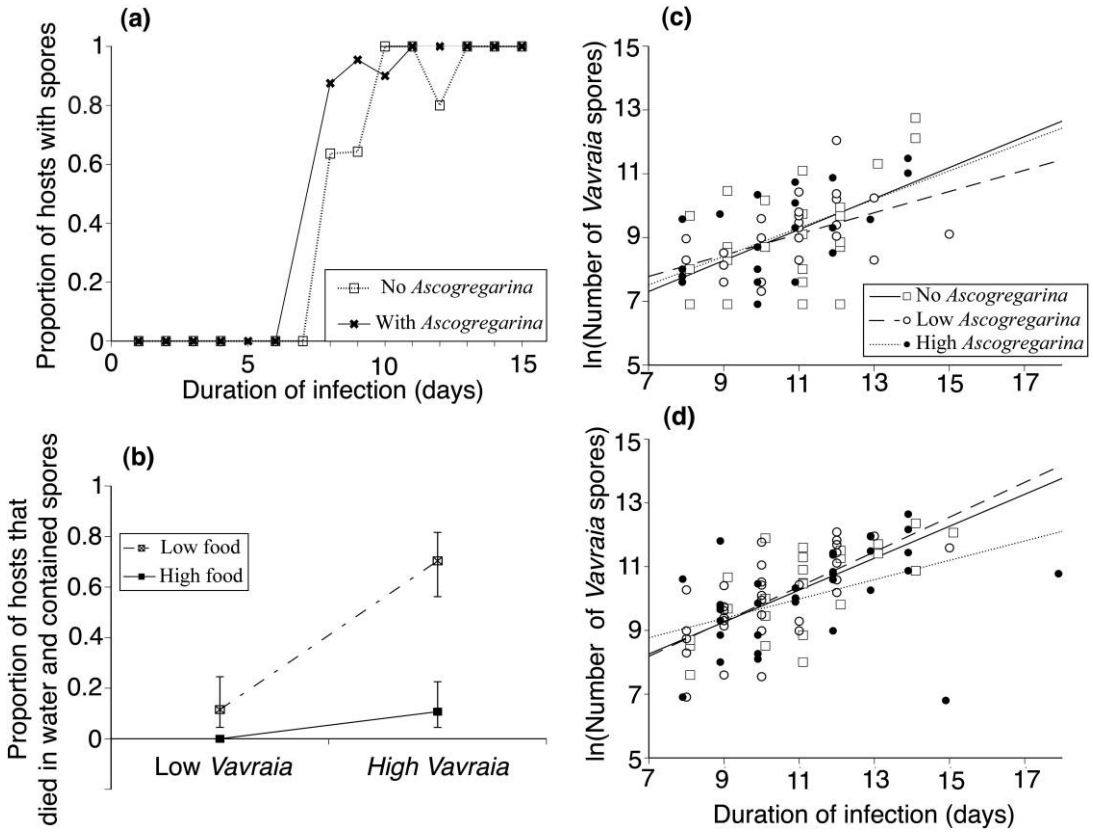

Figure 1: Effect of coinfection on the timing of the appearance of *Vavraia* spores (*a*), the proportion of mosquitoes that died in their larval site and contained spores (*b*), and the number of spores in the treatment with the low dose (*c*) and the high dose (*d*) of *Vavraia*. Vertical bars in *b* are confidence intervals. Key in *c* also applies to *d*. In *c* and *d*, each point represents a different mosquito.

We started with full factorial models and backward eliminated the nonsignificant terms, starting with the interactions of the highest order but keeping the marginally nonsignificant terms ($P < .1$). The statistical tables present the final models. When factors with more than two levels were significant, we used *t*-tests and χ^2 tests on the model's parameters to disentangle the effects of infectious dose and parasite presence. We also used contrasts between factor levels to test particular hypotheses about the effects of parasite dose.

All analyses were carried out with the statistical software JMP 6.0.3.

Results

Vavraia culicis

Presence of Spores. The likelihood of finding *Vavraia* spores increased with the duration of the infection; 10 days after infection, more than 90% of the mosquitoes contained spores (table 1; fig. 1*a*). Spores appeared slightly earlier in the presence of *Ascogregarina* than in its absence, irrespective of its dose (tests on the model parameters: no *Ascogregarina* vs. low *Ascogregarina*, $\chi^2 = 7.15$, $P = .008$; low *Ascogregarina* vs. high *Ascogregarina*, $\chi^2 = 0.47$, $P = .49$). Thus, if *Vavraia* were alone, 12 out of the 14 assayed hosts contained spores 8 days after infection, but in the presence of *Ascogregarina,* only 7 out of the 11 assayed hosts did.

Transmission within the Larval Site through Host Death. The proportion of hosts that harbored *Vavraia* spores and died before the adult stage (i.e., the parasite's success at transmitting within a breeding site) increased with increasing *Vavraia* dose and with decreasing food level of its host (table 1; fig. 1*b*). The number of spores in these hosts was not significantly influenced by any of our experimental treatments (all $P > .1$; mean 76,100 spores, SE 11,000).

Number of Spores. The number of *Vavraia* spores in the spore-positive mosquitoes increased with the duration of

Table 2: Results of the analyses for the presence and number of *Ascogregarina culicis* oocysts and for its transmission mode

Trait and factors	df	SS	χ^2	F	P
Oocyst presence:					
Vavraia	2		3.89		.145
Food	1		3.56		.0591
Vavraia × food	2		14.4		.0007
Oocyst number:					
Ascogregarina	1	2.69		3.68	.0569
Vavraia	2	10.3		7.10	.0011
Food	1	8.60		11.8	.0008
Error	144				
Proportion of oocysts transmitted locally:					
Vavraia	2	.742		3.40	.0360
Food	1	1.386		12.7	.0005
Error	145	15.8			

Note: Final models after backward elimination of insignificant terms ($P > .1$) are presented.

the infection (table 1; fig. 1*c*, 1*d*). It also depended on the combination of *Vavraia* and *Ascogregarina* treatments, as shown by the significant three-way interaction between these two factors and the duration of the infection. In order to understand the details of this interaction, we analyzed separately the high-*Vavraia* and low-*Vavraia* treatments. At the lower *Vavraia* dose, infecting the host with either dose of *Ascogregarina* slowed the production of spores (tests on the model parameters for slope: no *Ascogregarina* vs. low *Ascogregarina*, $t = -3.38$, $P = .001$; low *Ascogregarina* vs. high *Ascogregarina*, $t = 1.56$, $P = .12$). When the dose of *Vavraia* was high, only the high dose of *Ascogregarina* slowed down the production of *Vavraia* spores (tests on the model parameters for slope: no *Ascogregarina* vs. low *Ascogregarina*, $t = -0.08$, $P = .94$; low *Ascogregarina* vs. high *Ascogregarina*, $t = -2.34$, $P = .022$). Two individuals of the high-*Vavraia* and high-*Ascogregarina* treatment contained considerably fewer spores than the others (fig. 1*d*); when they were removed from the analysis, the three-way interaction between *Ascogregarina* and *Vavraia* treatments and the duration of the infection remained significant ($F_{2,143} = 3.49$, $P = .033$). Removing these two points had an impact when the mosquitoes that received a high dose of *Vavraia* were analyzed separately. Then, the effect of increasing the dose of *Ascogregarina* was not significant anymore (low *Ascogregarina* vs. high *Ascogregarina*, $t = -0.36$, $P = .72$).

Ascogregarina culicis

Presence of Oocysts. Whether *Ascogregarina* produced any oocysts was influenced by the interaction between the host's food availability and the *Vavraia culicis* treatment (table 2; fig. 2*a*). In particular, *Ascogregarina* was least likely to produce oocysts when exposed to the higher dose of *Vavraia* in hosts reared with little food (contrast high *Vavraia* and low food vs. three other treatments with *Vavraia*; $\chi^2 = 14.7$, df = 1, $P < .001$). This treatment also induced the highest preadult mortality among the hosts (fig. 3), which suggests that the parasite had little transmission success because its hosts died before it had produced its oocysts. This suggestion is supported by an analysis including a factor that describes whether the host died before the adult stage. While this factor was highly significant ($\chi^2 = 127.4$, df = 1, $P < .001$), it led to insignificant effects of *Vavraia* ($\chi^2 = 1.32$, df = 2, $P = .52$) and its interaction with host food ($\chi^2 = 2.19$, df = 2, $P = .33$).

Number of Oocysts. The number of oocysts produced (in the infections that produced at least one) was lowered by the presence of *Vavraia* and the host's starvation (table 2; fig. 2*b*). Infecting the mosquitoes with *Vavraia* decreased the number of oocysts (tests on the model parameters: no *Vavraia* vs. low *Vavraia*, $t = -2.81$, $P = .006$), but there was no clear effect of the dose of *Vavraia* (tests on the model parameters: low *Vavraia* vs. high *Vavraia*, $t = -0.79$, $P = .43$).

Proportion of Oocysts Transmitted Locally. The proportion of *Ascogregarina* oocysts transmitted locally (i.e., within the breeding site of its host larva) was increased by *Vavraia* infection and starvation of the host (table 2; fig. 2*c*). The effect of *Vavraia* depended on its dose (tests on the model parameters: no *Vavraia* vs. low *Vavraia*, $t = -0.49$, $P = .63$; low *Vavraia* vs. high *Vavraia*, $t = -2.4$, $P = .018$). However, this effect was due to the preadult mortality induced by *Vavraia* infection, which restricts the transmission of oocysts to the larval site. When the factor describing whether the host survived to the adult stage was included in the model, it was highly significant ($F_{1,144} =$

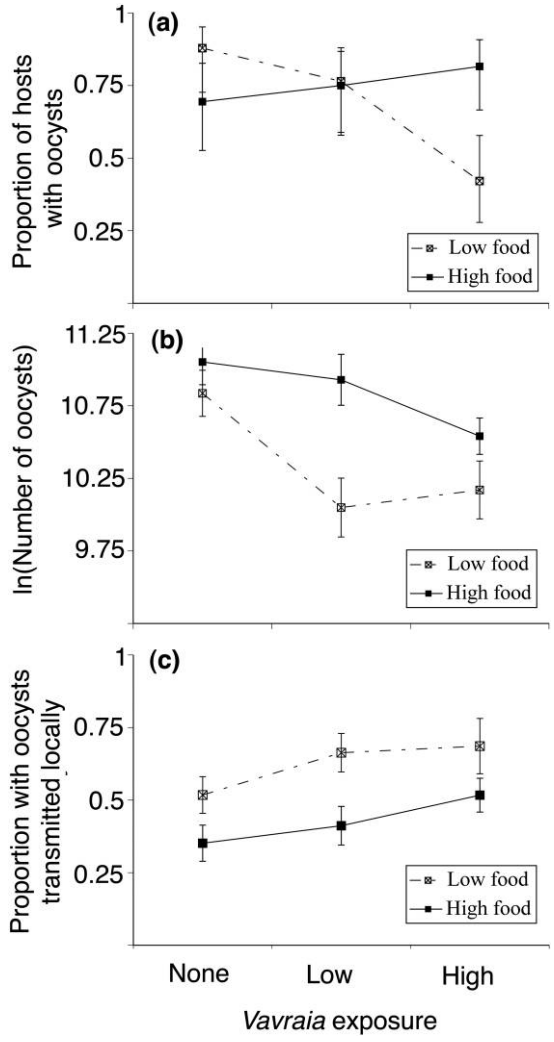

Figure 2: Effects of coinfection on the proportion of hosts where *Ascogregarina culicis* produced oocysts (*a*), the number of oocysts in those hosts that contained at least one oocyst (*b*), and the proportion of oocysts that were released locally (*c*). Symbols represent means, and vertical bars are confidence intervals in *a* and standard errors in *b* and *c*.

36, $P < .001$), whereas *Vavraia* infection became insignificant ($F_{2,144} = 1.34$, $P = .16$).

Host's Survival until Adulthood

The survival of the hosts until adulthood was reduced by *Vavraia* ($\chi^2 = 20$, df = 2, $P < .001$) and *Ascogregarina* ($\chi^2 = 6.26$, df = 2, $P = .044$; fig. 3). Host food availability interacted with *Vavraia* ($\chi^2 = 23.5$, df = 2, $P < .001$): the hosts infected with a high dose suffered highest mortality when in the low-food environment (contrast between this treatment and all other treatments: $\chi^2 = 68$, df = 1, $P < .001$). The significant role of *Ascogregarina* on mortality was due to its presence (contrast no *Ascogregarina* vs. low and high *Ascogregarina*, $\chi^2 = 5.59$, df = 1, $P = .018$; but no *Ascogregarina* vs. low *Ascogregarina*, $\chi^2 = 2.8$, $P = .094$) rather than its dose (low *Ascogregarina* vs. high *Ascogregarina*, $\chi^2 = 0.65$, df = 1, $P = .42$).

Discussion

We evaluated whether increasing the infectious dose of a parasite would increase its deleterious effect on a second parasite in its host. We used two pathogens of the mosquito *Aedes aegypti*, the gregarine *Ascogregarina culicis* and the microsporidium *Vavraia culicis*. For both parasites we found that a high dose induced stronger deleterious effects on the competitor than a low dose.

We expected that increasing the dose of *Vavraia* would enhance its performance and that increasing the dose of its competitor, *Ascogregarina*, would decrease it. Indeed, we observed that the rate at which *Vavraia* produces its spores depended on the interaction between the doses of the two parasites. At a high dose of *Vavraia*, only a high dose of its competitor reduced the rate of its spore production (fig. 1*c*); at a low dose of *Vavraia*, even a low dose of the competitor slowed the production of spores (fig. 1*b*). These results support our expectation.

Ascogregarina was affected in several ways by the competition with *Vavraia*. The proportion of hosts in which *Ascogregarina* produced some oocysts decreased if its competitor's dose increased and the host received little food (table 2; fig. 2*a*). This supports our prediction that dose effects are stronger when resources for the host are limiting. Local transmission was also highest when the dose of

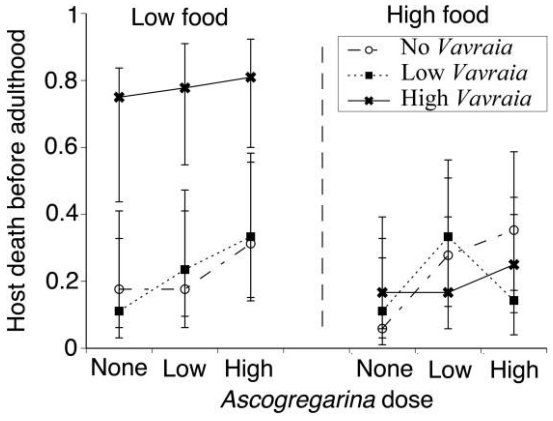

Figure 3: Effect of coinfection on the survival of the host until adulthood. Symbols represent means, and vertical bars are confidence intervals.

Vavraia was high and the host's food was scarce. This shows that, in addition to its effect on the intensity of the transmission, the competitor's dose can influence the mode of parasite's transmission.

These effects of *Vavraia* on *Ascogregarina* were mediated by the preadult mortality of the host. Mortality was highest when the dose of *Vavraia* was high and the amount of food was low (fig. 3), corroborating reports showing the high virulence of *Vavraia* in hosts reared in harsh environments (Bedhomme et al. 2004, 2005). In our experiment, many of the hosts that died before becoming adults were too young for *Ascogregarina* to have produced its oocysts. These results illustrate the conflict between the transmissions of our two parasites. A similar situation was described in the coinfection of amphipods by nematodes and trematodes (Thomas et al. 2002). Such conflicts can lead to the evolution of defense mechanisms by some of the coinfecting parasites, as for vertically transmitted microsporidians that reduce the virulence of the horizontally transmitted acanthocephalans with which they share their host (Haine et al. 2005).

It is likely that *Ascogregarina* and *Vavraia* competed for the same resources. Fewer infectious forms (i.e., spores and oocysts) were produced by either parasite in the presence of the second one (fig. 1*c*, 1*d*; fig. 2*b*). Because both parasites infect the gut of the host, it is reasonable to assume that the nutrients extracted from this organ by one parasite, and the damages that it caused, reduced the resources available for the other parasite. Alternatively, the host's immune system could play a role in this competition if, for instance, increasing the dose of one parasite increased the immune activity against the second parasite.

The effects of infectious dose that we observed have consequences for the epidemiology of parasites sharing a host. Increasing the prevalence or the production of infectious forms of a given parasite, thereby increasing its dose, should increase not only the frequency of coinfections but also the parasite's detrimental effect on its competitor. However, theoretical models studying the influence of multiple infections on parasite coexistence generally assume that the effects of within-host competition do not depend on the epidemiological situation (Hochberg and Holt 1990; May and Nowak 1995; Van Baalen and Sabelis 1995; Mosquera and Adler 1998; Blyuss and Kyrychko 2005; Martcheva and Pilyugin 2006). We predict that if dose effects such as the ones we report here were included in models, epidemiological feedback could lead to situations of competitive exclusion of one of the parasites in situations not previously described and, in particular, that initial conditions could determine which parasite is excluded.

Conclusions

We showed that the outcome of coinfection can depend on the conditions of infection. The detrimental effects of each parasite on the competitor were mediated by various interactions between their doses and the amount of food available to the host. Since infectious dose depends on epidemiology, our results suggest that the outcome of within-host competition between parasites depends on their epidemiological situations. This could produce a feedback leading to the exclusion of one of the competitors.

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