

# Cost of co-infection controlled by infectious dose combinations and food availability

Simon Fellous · Jacob C. Koella

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**Abstract** To what extent the combined effect of several parasite species co-infecting the same host (i.e. polyparasitism) affects the host's fitness is a crucial question of ecological parasitology. We investigated whether the ecological setting can influence the co-infection's outcome with the mosquito *Aedes aegypti* and two parasites: the microsporidium *Vavraia culicis* and the gregarine *Ascogregarina culicis*. The cost of being infected by the two parasites depended on the interaction between the two infectious doses and host food availability. The age at pupation of the mosquito was delayed most when the doses of the two parasites were highest and little food was available. As infectious dose increases with the parasites' prevalence and intensity of transmission, the cost of being co-infected depends on the epidemiological status of the two parasite species.

**Keywords** Co-infection · Infectious dose · Virulence · Ecology · Epidemiology

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S. Fellous · J. C. Koella  
Division of Biology, Imperial College London,  
Silwood Park Campus, Ascot SL5 7PY, UK

S. Fellous  
Laboratoire de Parasitologie Evolutive, UMR 7103,  
UPMC, Paris VI, 7 quai St Bernard, 75252 Paris, France

*Present Address:*  
S. Fellous (✉)  
Institut des Sciences de l'Evolution, CNRS, UMR 5554,  
Université de Montpellier II, CC 065,  
34095 Montpellier Cedex 05, France  
e-mail: simonfellous@free.fr

## Introduction

Why hosts suffer from parasitic infection and how to reduce this burden are central questions of ecological parasitology. Co-infection by several parasite species (also called polyparasitism) can substantially modify the detrimental effects that each parasite alone would have on its host (Bonsall and Benmayor 2005; Cox 2001; Marzal et al. 2008). Depending on the host and parasite species and the experimental set-up, the presence of a second parasite can increase the host's fitness, decrease it or leave it unchanged relative to what would be expected if the detrimental effects of each parasite alone were simply accumulated (Cox 2001; Marzal et al. 2008; Pullan and Brooker 2008). When virulence (generally expressed as parasite-induced mortality) relates to parasite transmission, the effects of co-infection on virulence are thought to have considerable influence on virulence evolution (Brown et al. 2002; de Roode et al. 2005; Frank 1992; Gower and Webster 2005; Van Baalen and Sabelis 1995). Recently, Alizon (2008) showed that whether co-infection increases or decrease the overall cost to being infected is key to the parasites' evolutionary trajectories. Even in this model, it is assumed that for a given combination of parasites, co-infection will either increase, decrease or not change the cost to the host. Here, we tested this assumption and investigated how simple ecological factors of the experimental set-up—contagious doses and availability of food—affect the fitness cost of being co-infected.

The number of a parasite's infectious forms a host is exposed to (i.e. infectious dose) can affect numerous aspects of host–parasite interactions, including host fitness (Brunner et al. 2005; Regoes et al. 2002; Schmid-Hempel and Frank 2007). For example, increasing the infectious dose of a baculovirus given to caterpillars increases their mortality (Hochberg 1991). In the mosquito *Aedes aegypti*,

we tested whether the detrimental effects of the co-infection by two parasite species, the microsporidium *Vavraia culicis* and the gregarine *Ascogregarina culicis*, depend on the two infectious doses, expecting greatest effects when the doses were highest. The quality of the host's environment is another factor that is known to affect the effect of single infections on host phenotype (Bedhomme et al. 2004; Felous and Salvaudon 2009; Vale et al. 2008). But the influence of environmental quality in cases of co-infections is less well known (Thomas et al. 2003). In order to examine this question, we also varied the quantity of food given to the host larvae. Because reduced food availability means reduced available resources with which to fight the parasites, we predicted that costs of co-infection would be highest when habitat quality was poorest (Jokela et al. 2005).

## Materials and methods

### Biological system

The yellow fever Mosquito, *Aedes aegypti*, is widespread in many subtropical areas (Christophers 1960). The larvae develop in small water tanks where they feed on bacteria. After a minimum of about 7 days they transform into pupae, and emerge as adults 2 days later.

The microsporidium *Vavraia culicis* is an obligate intracellular parasite of several mosquito species (Andreadis 2007). The host larvae become infected when they ingest the spores of the parasite along with their food. In *A. aegypti*, infections have two possible outcomes. With little food or a high dose of spores, the infected larvae and pupae die. Their death allows *Vavraia* spores to be released and therefore new infections. Otherwise, the larvae survive and infected adults emerge. These infected adults are smaller and survive less long than uninfected adults (Michalakis et al. 2008). 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 extra-cellular parasite of *A. aegypti* (Reyes-Villanueva et al. 2003; Sulaiman 1992). Larvae are infected by ingesting the parasite's oocysts along with their food. The parasite has two modes of transmission (Roychoudhury and Kobayashi 2006). First, local transmission occurs when pupae or emerging mosquitoes that harbor oocysts die in the breeding site or when oocysts are released as adults emerge. 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. Except for some Asian strains, this parasite usually

has few deleterious effects on the host (Reyes-Villanueva et al. 2003; Sulaiman 1992).

The two parasite species initiate infection by piercing the gut of the host (Andreadis 2007; Chen 1999), creating a bottleneck where competition for resources is likely to occur. Besides, there is 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. This, in addition to the possibility of infecting simultaneously the host with the two species of parasites, made this system ideal for investigating the competitive interactions induced by co-infection.

J. J. Becnel from the United States Department of Agriculture established the mosquito and *Vavraia* strains, which he had isolated from natural populations in Florida. The *Ascogregarina* strain was obtained from an American population of mosquitoes by Dawn 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 treatments. For each of them, we reared 18 larvae individually (i.e. 18 independent replicates) in 12-well plates. The positions of the individual replicates from each treatment were organized by blocks so that the individuals receiving the same treatments would be evenly distributed among the rearing plates.

On the first day of the experiment, we synchronously hatched several hundred mosquito eggs under low pressure. The following day, each larva was placed into a well of 12-well tissue culture plates with 4 ml of deionized water. Two-day-old larvae were exposed to 0 (no *Ascogregarina*), 500 (low *Ascogregarina*) or 5,000 *Ascogregarina* oocysts (high *Ascogregarina*) and 0 (no *Vavraia*), 1,000 (low *Vavraia*) or 10,000 *Vavraia* spores (high *Vavraia*). These doses usually ensure prevalence higher than 90% in single infections (S. F., personal observation). Exposure to the two parasites was simultaneous. All larvae were fed ad libitum during the first 24 h. The larvae reared in the high food treatment received 0.04 mg fish food (Tetramin)/larva on the 2nd day, 0.08 mg/larva on the 3rd day, 0.16 mg/larva on the 4th day, 0.32 mg/larva on the 5th day, 0.64 mg/larva on the 6th day and 0.32 mg/larva on each of the following days. The larvae reared at low food received half of these amounts. The food quantity of the high food treatment allows rapid development and large adults; the low food treatment reduces these traits to values more frequent in natural settings (Christophers 1960).

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 frozen at  $-20^{\circ}\text{C}$  until they were further evaluated. In order to estimate adult size, which correlates with fecundity in females (Christophers 1960), one wing per individual was removed, mounted on a slide and measured from the allula to the peripheral tip of vein R3. We checked the mosquitoes for the presence of oocysts and spores using a haemocytometer and a phase-contrast light microscope ( $\times 400$ ). Prevalence was 100% for all infectious treatments. The present paper strictly focuses on the effect of co-infection on a host's phenotype; the results for parasite transmission are accordingly detailed in a separate article (Fellous and Koella 2009).

The experiment was performed in a room at  $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ,  $60 \pm 10\%$  humidity and 12 h light per day.

### Statistical analyses

To estimate host fitness we analysed age at pupation (in days), the proportion of mosquitoes that successfully emerged as adults and wing length (an estimator of size and fecundity).

We used linear models for age at pupation and wing length, and generalized linear models for the proportion of hosts surviving to adulthood (using binomial errors and controlling for overdispersion). These models contained the two parasite species (with three levels of each: no parasites, low dose, high dose) as ordinal factors and food level as a nominal factor. We also included the sex of the host in the analyses of age at pupation and wing length. We started from full factorial models and backward eliminated the insignificant terms ( $P > 0.1$ ), starting with the interactions of highest order. Insignificant terms remained in the model if they were involved in significant interactions of higher order. When a dose (which has more than two levels) was significant, we used contrasts to disentangle the effects of infectious dose and parasite presence. We checked for homoscedasticity and other assumptions of the models. All analyses were carried out with the statistical software JMP 6.0.3.

## Results

### Age at pupation

The age at pupation of the host was controlled by the three-way interaction between food quantity, *Ascogregarina* and *Vavraia* (Table 1; Fig. 1a). The mosquitoes that received little food and high doses of each parasite developed most slowly (contrast analysis between this treatment and the other co-infection treatments at low food,  $F_{1,213} = 25.1$ ,

$P < 0.0001$ ). In the low food treatment average age at pupation across all treatments was 12.1 days (SE 0.1), but was delayed to 13.6 days (SE 0.3) when the two doses were high. Average age at pupation was 8.7 days (SE 0.1) in the high food treatment. The analysis of age at pupation with a survival analysis or an ordinal logistic regression (after coding the response variable as ordinal) gave identical results.

### Host's survival until adulthood

Host's survival up to adulthood was reduced by *Vavraia* and *Ascogregarina* (Table 1; Fig. 1b), but their interaction was not significant ( $\chi^2 = 2.27$ ,  $df = 4$ ,  $P = 0.69$ ). Food availability interacted with *Vavraia*: the hosts infected with a high dose of spores suffered high mortality when in the low food environment (78 mortality vs. 11% in the other treatments; contrast between this treatment and all other treatments,  $\chi^2 = 68$ ,  $df = 1$ ,  $P < 0.0001$ ). The significant role of *Ascogregarina* on mortality resulted from its presence (contrast no *Ascogregarina* vs. low and high *Ascogregarina*,  $\chi^2 = 5.59$ ,  $df = 1$ ,  $P = 0.018$ ) rather than its dose (low *Ascogregarina* vs. high *Ascogregarina*,  $\chi^2 = 0.65$ ,  $df = 1$ ,  $P = 0.42$ ). Almost half of the mosquitoes (47/98) that died before adulthood had already pupated and many were in the process of emerging. They thus provided data on the timing of pupation while the sex of many of them could be recorded. Even when mortality was greatest (i.e. with little food and a high dose of *Vavraia* spores), only 10% of the mosquitoes died prior to pupating.

### Host's wing length

The host's wing length was affected by *Ascogregarina*, *Vavraia*, host sex and the amount of food it received, but not by any interaction between the two parasites (Table 1; Fig. 1c). The wings of females measured, on average, 3.4 mm and those of males 2.8 mm.

## Discussion

The duration of the host's development was affected by an interaction between the dose of each of the two parasites and the amount of food it received. Thus host fitness was affected by the interplay of these ecological factors.

This interactive effect of infectious doses on age at pupation is the main finding of this study and is important for two reasons. First, age at pupation affects generation time, and therefore has a large influence on fitness (Stearns 1992). Second the interaction between the doses of the two parasites may have consequences for host populations when parasite prevalences are high. As infectious dose is

**Table 1** Final statistical models (after backward elimination of insignificant factors) for the analyses of age at pupation, survival until adulthood and wing length

Trait	Factors	df	SS	F	P-value
Age at pupation	<i>Vavraia</i>	2	6.27	6.27	0.0022
	<i>Ascogregarina</i>	2	3.35	3.35	0.0366
	Food	1	79.7	159	<0.0001
	Host's sex	1	72.3	144.8	<0.0001
	<i>Vavraia</i> × <i>Ascogregarina</i>	4	11.0	5.52	0.0003
	<i>Vavraia</i> × Food	2	1.01	1.01	0.3649
	<i>Ascogregarina</i> × Food	2	1.82	1.82	0.1642
	<i>Vavraia</i> × <i>Ascogregarina</i> × Food	4	6.53	3.27	0.0125
Error		215	107		
				$\chi^2$	P value
Survival to adulthood	<i>Vavraia</i>	2		20.0	<0.0001
	<i>Ascogregarina</i>	2		6.26	0.0438
	Food	1		0.002	0.9637
	<i>Vavraia</i> × Food	2		23.5	<0.0001
				F	P value
Wing length	<i>Vavraia</i>	2	0.235	10.9	<0.0001
	<i>Ascogregarina</i>	2	0.073	3.4	0.0353
	Food	1	0.348	32.5	<0.0001
	Host's sex	1	5.680	530	<0.0001
	Host's sex × <i>Ascogregarina</i>	2	0.068	3.17	0.0439
	<i>Vavraia</i> × Food	2	0.073	3.43	0.0345
	Host's sex × Food	1	0.070	6.58	0.0111
	Error		188	2.014	

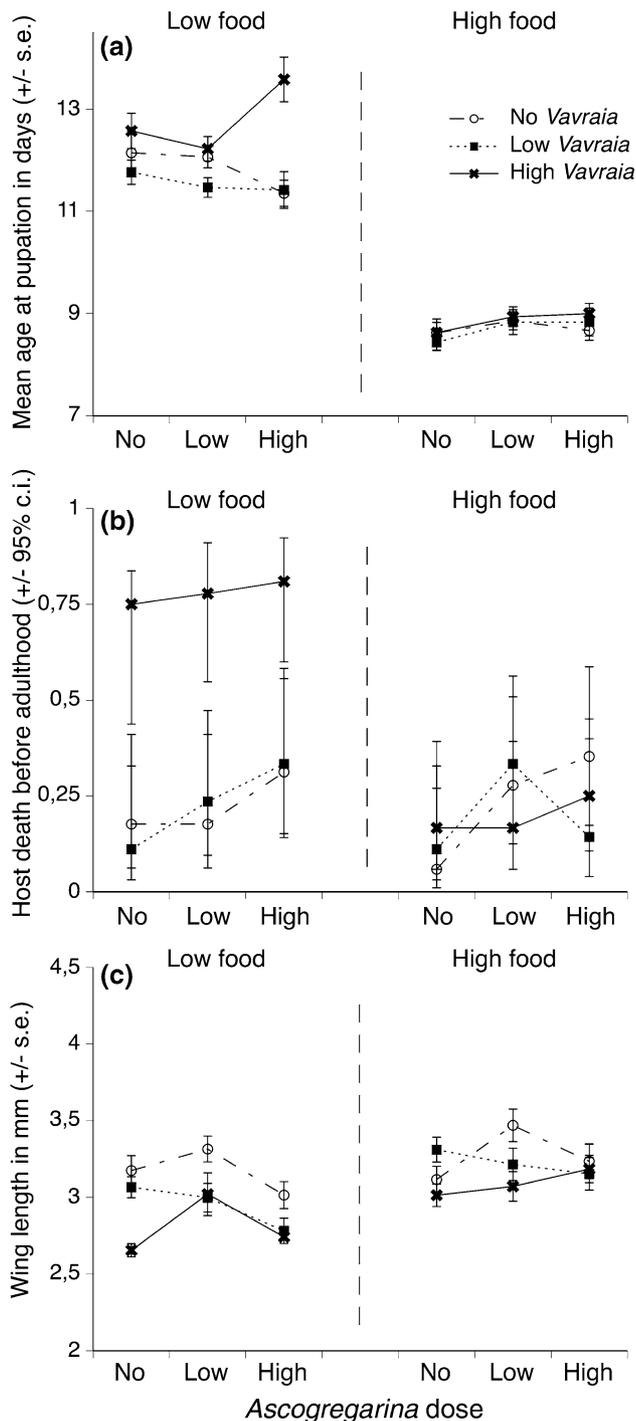
determined by the parasite's density and transmission, dose depends on its epidemiology. The combined effect of the doses of the two co-infecting parasites therefore suggests that the detrimental effects of a co-infection can depend on the details of the parasites' epidemiologies. This expands on the more usual idea that the parasites' epidemiologies influence the host population by determining the *frequency* of co-infection (Pullan and Brooker 2008). Here we show that a more subtle influence of epidemiology can be important, through infectious-dose effects.

The effect of the combination of the parasite doses was only apparent when the hosts received little food and doses were highest. In other cases the infection by the two parasites had little effect on the host's development (Fig. 1a). This is in agreement with, and expands on, previous reports on *Vavraia*, which show more detrimental effects when host food is limiting than when it is abundant (Bedhomme et al. 2004). That parasite doses only interacted in the low food treatment not only highlights the importance of environmental quality for a host's resistance or tolerance to infection (Jokela et al. 1999; Lambrechts et al. 2006; Vale et al. 2008), it also shows that co-infections can have effects that are only revealed in specific environments and suggests that these effects might be negligible in others. This observation

is in contradiction with even the most recent models of parasite virulence evolution in which a specific combination of parasites always has the same type of effect on host fitness (Alizon 2008; Brown et al. 2002; Frank 1992).

The three phenotypic traits of the host that we measured responded differently to our treatments. A statistical interaction between the two parasites was only found for the duration of larval development. But mortality before adulthood was only mildly affected by the presence of *Ascogregarina* (Fig. 1b). This observation is in agreement with previous findings on the moderate virulence of this species (Reyes-Villanueva et al. 2003). No clear pattern emerged from the analysis of wing length and there was no statistical interaction between the two parasites. The heterogeneity of results among traits illustrates the importance of considering multiple traits when attempting to estimate fitness (Voordouw et al. 2009).

It is tempting to compare the effects of co-infection on the host with the ones on the parasites that we reported earlier (Fellous and Koella 2009). Interestingly, the development of the parasites and that of the host were rarely affected by infectious dose and food availability in the same fashion. For example, *Vavraia* spore production was not particularly reduced when host development was slowest



**Fig. 1** The effects of host food quantity and infectious doses of *Vavraia culicis* and *Ascogregarina culicis* on **a** age at pupation (mean estimated from raw data), **b** proportion of mosquitoes that die before emerging as adults and **c** wing length (mean estimated from raw data). Note the elevated age at pupation of the mosquitoes reared with little food and that received high doses of *Vavraia* and *Ascogregarina* (**a**, left)

(i.e. with low food and high doses of parasites). However, host mortality induced by *Vavraia* infection directly constrained *Ascogregarina* oocyst production: in the low food–

high *Vavraia* treatment, many hosts died before the oocysts were produced. In all cases, infectious dose appeared to be an important factor. This underlines the key role of dose in infectious processes, showing that, as in single-species infections (Brunner et al. 2005; de Roode et al. 2007; Ebert et al. 2000), this parameter can be crucial to co-infections.

To conclude, the effect on the host of the co-infection by our two parasite species depended on the interplay of three ecological factors, namely the infectious dose of each parasite and the host's food availability. Our study thus shows that the detrimental effect of a co-infection by two parasites is not a fixed parameter, but depends on the epidemiological context and the quality of the host's habitat.

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