Received: 3 May 2019 | Accepted: 17 June 2020

DOI: 10.1111/1365-2656.13302

RESEARCH ARTICLE

Journal of Animal Ecology | BRITISH ECOLOGICAL SOCIETY

Sequential co-infections drive parasite competition and the outcome of infection

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Handling Editor: Isabella Cattadori

Abstract

1. Co-infections by multiple parasites are common in natural populations. Some of these are likely to be the result of sequential rather than simultaneous infections. The timing of the co-infections may affect their competitive interactions, thereby influencing the success of the parasites and their impact on the host. This may have important consequence for epidemiological and eco-evolutionary dynamics.
2. We examined in two ecological conditions the effect of sequential co-infection on the outcome of infection by two microsporidians, *Vavraia culicis* and *Edhazardia aedis*, that infect the mosquito *Aedes aegypti*. The two parasites have different transmission strategies: *V. culicis* is transmitted horizontally either among larvae or from adults to larvae, while *E. aedis* can be transmitted horizontally among larvae or vertically from females to their eggs.
3. We investigated how the timing and order of the co-infection and how the host's food availability affected the parasite's transmission potential (the percentage of individuals that harboured transmissible spores) and the host's juvenile survival, its age at emergence and its longevity.
4. The outcome of co-infection was strongly affected by the order at which the parasites arrived. In co-infections, *V. culicis* had greater horizontal transmission if it arrived early, whereas the transmission potential of *E. aedis*, either vertical or horizontal, was not affected by the competitor *V. culicis*. The availability of food determined the duration of infection leading to variation in mortality and in the transmission potential. For both parasites low food decreased juvenile survival, delayed emergence to adulthood and increased horizontal transmission potential. High food increased juvenile survival and the probability of emergence with higher vertical transmission for *E. aedis*. Overall, our results suggest that early infection favours transmission and that (a) *V. culicis* plastically responded to co-infection, (b) *E. aedis* was not affected by co-infection but it was more susceptible to factors extending or decreasing the time it spent in the host (time of infection and food).
5. Our results emphasize the complexity of the impact of co-infection on host–parasite interactions. In particular, the timing and order of sequential co-infections can result in different within-host dynamics and modify infection outcomes.

KEYWORDS
horizontal transmission, host–parasite interactions, microsporidia, multiple infections, priority effects, timing of infection, vertical transmission, virulence

1 | INTRODUCTION

Hosts are often infected by several strains or species of parasites (Lagrue, McEwan, Poulin, & Keeney, 2007; López-Villavicencio et al., 2007; Malpica, Sacristán, Fraile, & García-Arenal, 2006; Rutrecht & Brown, 2008; Turner & Duffy, 2008). Co-infections have important consequences for epidemiology and evolution, for they can lead to different outcomes for both the hosts and the parasites than any of the individual parasites (Alizon, 2013; Alizon, de Roode, & Michalakis, 2013; Mideo, 2009; Rodrigues, Duncan, Clemente, Moya-Laraño, & Magalhães, 2016; Tollenaere, Susi, & Laine, 2016). For instance, co-infections can increase the mortality rate of the host, so that higher levels of virulence evolve (Levin & Bull, 1994). The within-host interactions between parasites can regulate their coexistence, with mutually beneficial, antagonistic or neutral impacts on one another (Vasco, Wearing, & Rohani, 2007), leading to higher (Susi, Barrès, Vale, & Laine, 2015) or weaker (Duncan, Agnew, Noel, & Michalakis, 2015) production of transmission stages. Nevertheless, co-infecting parasites typically have conflicting interests, for they compete for the exploitation of the same host for their growth and transmission (Bell, de Roode, Sim, & Read, 2006; Ben-Ami, Rigaud, & Ebert, 2011; Wargo, de Roode, Huijben, Drew, & Read, 2007). The mechanisms of this competition include the limitation of the host's resources for the parasite's growth and the host's cross-immunity.

Two main factors that may influence co-infection dynamics, and that thus deserve more attention, are the timing of each infection and the order with which parasites infect the host (Ben-Ami, 2019; Karvonen, Jokela, & Laine, 2019). Indeed, the time at infection, corresponding to parasites infecting a host at different ages, can influence interaction traits, including host susceptibility, virulence and transmission. For example, younger beetles or crustaceans are more susceptible to infection compared to older individuals (Blaser & Schmid-Hempel, 2005; Izhar & Ben-Ami, 2015). This, and other effects of time at infection, have been widely observed in many invertebrates and may have important consequence for disease dynamics, epidemiology and evolution (Ben-Ami, 2019). Similarly, in several plants and animals, the order of infection in sequential co-infections alters infection pattern and determines the outcomes of the infection (Clay, Dhir, Rudolf, & Duffy, 2019; Hoverman, Hoye, & Johnson, 2013; Laine, 2011; Natsopoulou, McMahon, Doublet, Bryden, & Paxton, 2015; Sandoval-Aguilar et al., 2015), which can affect ecology by influencing the population density of the host and the intensity of epidemics, and it can affect evolutionary by influencing the parasite's investment in its transmission (Halliday, Umbanhowar, & Mitchell, 2017; Marchetto & Power, 2018; Wuerthner, Hua, & Hoverman, 2017). In most cases, the first parasite has an advantage (Karvonen et al., 2019, but see also Clay, Cortez, Duffy, & Rudolf, 2019) by, for example, depleting the resources of the host and thereby outcompeting a second parasite (Hoverman et al., 2013). In other cases, however, the first parasite may facilitate a second one by compromising the immune response and thereby increase susceptibility of the host (Rolff & Siva-Jothy, 2003).

An additional factor underlying the competition among co-infecting parasites is that they might have different modes of transmission, with a different balance between vertical and horizontal transmission (Jones, White, & Boots, 2010). Conflicting transmission modes may lead to different host exploitation and higher virulence (Ben-Ami et al., 2011). Alternatively, vertically transmitted parasites (transmission from mother to offspring) may protect their host against further infections (Hedges, Brownlie, O'Neill, & Johnson, 2008; Scarborough, Ferrari, & Godfray, 2005) as their transmission is coupled with lifetime reproductive success of the host (Frank, 1996). These within-host interactions are also likely to be affected by food (Wolinska & King, 2009): direct effect with competition for resources as mentioned, and indirect effect as food also influences immune response (Lee, Simpson, & Wilson, 2008). Finally, food can affect transmission mode. Getting a lot of resources, for example, lets hosts have many offspring and vertical transmission beneficial (Agnew & Koella, 1999; Restif & Kaltz, 2006).

Here using the mosquito *Aedes aegypti* and two parasites, the microsporidians *Vavraia culicis* and *Edhazardia aedis*, we asked how the timing and order of infection and the amount of food available to the larvae affect the outcome of the infection. We chose these parasites for several reasons. First, they have different transmission strategies (Desjardins et al., 2015). Both parasites are transmitted horizontally when infected larvae die and release spores into the water, which are then ingested by other larvae. *V. culicis* has some additional horizontal transmission from adults to larvae, when its spores are released from adults that die in the water or when they are laying eggs. *E. aedis* has vertical transmission in addition to its horizontal transmission. Its two transmission routes involve specialized types of spores: binucleate spores infect eggs for vertical transmission, and uninucleate spores are transmitted horizontally. Second, the amount of food available to the larvae greatly affects the infection dynamics of both parasites; better fed larvae develop more rapidly, are more likely to survive to become adults and (for *E. aedis*) the parasites invest more in vertical and less in horizontal transmission (Zilio, Thiévent, & Koella, 2018). Third, although the immune system is stimulated (Biron et al., 2005), no immune response is known to be effective against the parasites. We can therefore concentrate on resource competition as the main mechanism of the interactions within the host. We had several expectations for the outcome of infection of our host–parasite system.

1.1 | Co-infection

Co-infections are expected to be more virulent than single infections. Since they are more likely to kill larvae and reduce juvenile survival, there is less opportunity for vertical transmission, so the parasites should invest more in horizontal transmission among larvae. Since co-infections use more resources than single infections, co-infected adults emerge with fewer resources and should die earlier (Duncan et al., 2015). These effects are expected to be stronger when food is more limiting.

1.2 | Order of co-infection

Since there is little evidence for an effective immune response of mosquitoes against microsporidians (Biron et al., 2005; Desjardins et al., 2015), we do not expect that the first infection will facilitate the second, but that the first infection has an advantage (Hood, 2003; Hoverman et al., 2013; Karvonen et al., 2019). In order to avoid competition with the second parasite it would invest more in horizontal transmission, and thus have less vertical transmission, than when it infects its host alone. The second (outcompeted) parasite, in contrast would be suppressed, so obtain less horizontal and less vertical transmission than when it infects its host alone. If the first parasite suppresses the second by using up resources before the second infection (Duncan et al., 2015; Rivero, Agnew, Bedhomme, Sidobre, & Michalakis, 2007), we expect that the effect of timing is stronger if the mosquitoes are less well fed.

1.3 | Food and timing of single infections

One of the conclusions of the studies mentioned above on single infections is that the duration of infection in larvae determines the survival of larvae and the potential of the parasite for horizontal transmission. Therefore in single infections, low food and early infection, which both slow growth and thus delay emergence, should increase horizontal transmission, while high food and late infection should increase juvenile survival and increase vertical transmission (Bedhomme, Agnew, Sidobre, & Michalakis, 2004; Zilio et al., 2018). However, we had no expectation for the effects of food or timing of infection on the longevity of infected hosts, for they affect, on the one hand, the resources available for the parasite's growth and, on the other hand, the quality of the host and its ability to fight the parasite.

2 | MATERIALS AND METHODS

2.1 | Mosquitoes

We used the UGAL strain of the mosquito *A. aegypti*, provided by Patrick Guérin (University of Neuchâtel). Our colony is maintained at 26°C, with 70% humidity and a 12-hr light and 12-hr dark regime. In each generation, we keep 1,500 adult mosquitoes and give them constant access to a 10% sucrose solution.

2.2 | Microsporidia

Edhazardia aedis and *V. culicis* are microsporidians, a group of intracellular parasites that are common in insects. Both are natural parasites of *A. aegypti* and were provided by J. J. Becnel (USDA, Gainesville, USA). *Edhazardia aedis* is specific to *A. aegypti* (Becnel & Johnson, 1993), whereas *V. culicis* can infect several genera of mosquitoes (Andreadis, 2007).

The life cycle of *E. aedis* involves horizontal and vertical transmission (Andreadis, 2007) with morphologically different types of spores: uninucleate spores for horizontal transmission and binucleate spores for vertical transmission. The binucleate spores can infect the oocytes of a female. Once these vertically infected eggs hatch and the larvae emerge, the parasite continues its development and eventually produces a generation of uninucleate spores. These are released from dead larvae and are horizontally transmitted to other larvae that ingest them. After a period of development in the horizontally infected mosquito, the parasite produces a new generation of binucleate spores. If these are produced within an adult female, the next round of vertical transmission is started. If, however, they are produced before the mosquito emerges, the parasite's development can continue to produce another generation of uninucleate spores that can kill the larva, giving a second round of horizontal transmission and bypassing vertical transmission. Thus, depending on conditions, the parasite can either alternate vertical and horizontal transmission or it can go through subsequent rounds of horizontal transmission. Note that males provide no opportunity of vertical transmission, and that vertical and horizontal transmission from the same host individual is not possible, for horizontal transmission requires the host's death.

Vavraia culicis infects larvae when they ingest the spores. The spores enter the gut cells, from where the infection spreads to the fat body and other tissues (Andreadis, 2007). The microsporidia are transmitted horizontally when the infected larvae, pupae or adults die and release the spores into the water (Becnel, White, & Shapiro, 2005). Spores can also be transmitted from egg-laying females to larvae, for spores in infected females adhere to their eggs. To distinguish this horizontal transmission from the transmission among larvae and from the vertical transmission of *E. aedis*, we will call this 'female-to-larva transmission'.

2.3 | Experimental design

The experiment was performed in the standard rearing conditions of the colony: 26°C, 70% relative humidity and a 12-hr light and 12-hr dark regime. To assess the effects of co-infection, order of infection and amount of food, we reared mosquito larvae in one of two feeding

regimes, exposed them to one of the parasites at 1 of 2 days, and either exposed them or not to the other parasite on the other day. We then measured three life-history traits—juvenile survival, emergence and longevity—and the parasite's development to evaluate the potential for transmission among larvae and from adults to larvae. Note that since the main questions were about sequential co-infections and transmission, we did not consider uninfected mosquitoes or simultaneous co-infections.

We rehydrated eggs of our colony in 100 ml of deionized water and hatched them synchronously at low atmospheric pressure. About 1,200 larvae were haphazardly transferred to and reared individually in 12-well tissue-culture plates containing 3 ml of deionized water. Larvae were reared either with high or low food (100% or 50% of the standard diet in our laboratory). Within each food level, larvae were exposed to *V. culicis* early or late (i.e. 2 or 4 days after hatching), to *E. aedis* early or late, or to both parasites where either *V. culicis* was early and *E. aedis* was late or *V. culicis* was late and *E. aedis* was early. Since this gives 12 treatments, each well on the 12-well plates represented a different treatment. The high food regime was at age 0:0.06 mg of TetraMin™ fish food per larva, age 1: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg and age 5 and older: 0.32 mg.

We obtained the spores of *V. culicis* from infected adults killed 10 days after emergence, and the spores of *E. aedis* from vertically infected larvae killed 7 days after hatching. For both parasites, we crushed and homogenized 20 mosquitoes in an Eppendorf tube containing 1 ml of deionized water. The concentration of the spores was determined with a haemocytometer and a phase contrast microscope (Zeiss Axio Lab.A1). The solution was then diluted to obtain the concentration we used in the experiment, 10^4 spores of *V. culicis* in 100 μl and 400 uninucleate spores of *E. aedis* in 100 μl. Our earlier studies have shown that these densities of spores assure high infection rates and prevent excessive mortality rates. To ensure that an observed effect of co-infection was not due to the additional nutrition derived from crushed mosquitoes, we added 100 μl of a solution of crushed uninfected larvae to the wells containing singly infected larvae on the appropriate day.

We transferred the pupae individually to Falcon tubes and provided the emerging adults with a cotton ball soaked with 10% sugar solution, which we changed every 6 days. The survival of all individuals (larvae, pupae and adults) was checked every 24 hr throughout the experiment. Dead individuals were moved to a 2 ml plastic tube and stored at −20°C until further investigation. The experiment was stopped 24 days after hatching and all the individuals alive at that time were moved to a freezer at −20°C. We counted the parasite's spores in each mosquito under a phase contrast microscope (Zeiss Axio Lab.A1) after adding 0.1 ml of deionized water to the tubes, homogenizing the samples using a TissueLyser LT—QIAGEN beads machine, placing 8 μl of the obtained solution on a haemocytometer. The treatments of the samples were unknown while we counted the spores. Note that although we counted the spores, we later analysed only the presence or absence of spores, for since few mosquitoes contained uninucleate spores of *E. aedis* that any quantitative analysis of the number of spores would have been strongly biased by the mosquitoes without any spores.

2.4 | Statistical analyses

All statistical analyses were carried out with R (version 3.6.3, R Core Team, 2020). We used the SURVIVAL package (Therneau & Grambsch, 2000) for the Cox model. Significance tests were based on type 3 tests, calculated with the CAR package (Fox & Weisberg, 2019).

2.4.1 | Parasite transmission

For *V. culicis* we considered two routes of horizontal transmission. We defined the potential for transmission among larvae as the percentage of individuals that died as juveniles that harboured spores and defined the potential of female-to-larva transmission as the percentage of mosquitoes that survived to become adult females and harboured spores. For *E. aedis*, we defined the potential for horizontal transmission among larvae as the proportion of individuals that died as juveniles and harboured uninucleate spores. For vertical transmission we analysed the proportion of mosquitoes that emerged as females and harboured binucleate spores. We ran a separate model for each parasite and for each transmission route. We therefore ignored the data with single infections by *E. aedis* when we analysed the transmission of *V. culicis*, and we ignored the data with single infections by *V. culicis* when we analysed the transmission of *E. aedis*. For each analysis of transmission potential we used a glm with binomial distribution that included the time of infection, whether mosquitoes were co-infected or not, the amount of food and the two-way interactions as nominal factors.

2.4.2 | Host life history

We wanted to know whether the host's life-history traits were affected by the parasite in single infections and whether this effect was modified by co-infections, the order of co-infections and the food available to the larvae. We therefore combined the data for the two parasites and defined a new variable—'focal parasite'. For singly infected mosquitoes, the focal parasite was the parasite used for the infection. For co-infected mosquitoes, we randomly assigned half of the co-infections to *V. culicis* and the other half to *E. aedis* as focal parasite. This procedure enabled us to evaluate the effect of co-infection on the life-history relative to the effects of single infections by *V. culicis* and *E. aedis*. We analysed juvenile survival with a generalized linear model (glm) with binomial distribution, the age at emergence of females with a Cox proportional hazards regression model, and female longevity with a Cox proportional hazards regression model censoring the mosquitoes

killed at 24 days after hatching (when more than half of the mosquitoes had died (59%) and we ended the experiment). Each analysis included the focal parasite, the time of infection by the focal parasite, the presence or absence of a co-infection, the amount of food and all interactions as nominal factors. Since co-infected mosquitoes were assigned randomly to each focal parasite, we ensured that our results were not due to a sampling artefact by repeating these steps 100 times. We evaluated the strength of each factor with the proportion of the 100 repeats that the factor was significant (at the 0.05 level) and with the mean *p*-value. Note that the plots do not include 'focal parasite', but simply show the data of single infections and of co-infections with *V. culicis* before *E. aedis* (Vav First in Figures 3–5) or *E. aedis* before *V. culicis* (Ed First in Figures 3–5).

3 | RESULTS

3.1 | Parasite transmission

3.1.1 | *Vavraia culicis*: Among larvae transmission

Co-infection increased the potential for transmission of *V. culicis* among larvae; more of the co-infected mosquitoes (5.4%, CI 3.53–8.20) died before emergence and harboured spores than of those infected only with *V. culicis* (1.9%, CI 0.94–3.93; $\chi^2 = 11.86$, $df = 1$, $p < 0.001$; Figure 1a). The potential for transmission among larvae was higher after early infection (5.5%, 95% confidence interval 3.58–8.33) than after late infection (1.9%, CI 0.92–3.86; $\chi^2 = 5.22$, $df = 1$, $p = 0.023$). This was in particular the case for co-infected individuals, in which *V. culicis* had 7% (CI 4.12–11.65) transmission potential if it was the first infection and 3.9% (CI 1.91–7.85) if it was the second (interaction co-infection × timing: $\chi^2 = 4.37$, $df = 1$, $p = 0.03$). The potential of transmission among larvae was higher in mosquitoes reared at low food (6.2%, CI 4.18–9.15) than high food (1.1%, CI 0.43–2.80; $\chi^2 = 10.23$, $df = 1$, $p = 0.001$). The amount of food had no significant impact on how co-infection ($\chi^2 = 1.82$, $p = 0.177$) or the timing of infection ($\chi^2 = 0.61$, $p = 0.436$) affected transmission.

3.1.2 | *Vavraia culicis*: Female-to-larva transmission

In contrast, co-infection by *E. aedis* decreased the potential transmission of *V. culicis* from adult females to larva from 13.5% (CI 10.39–17.45) to 10.8% (CI 8.04–14.39; $\chi^2 = 4.3$, $df = 1$, $p = 0.038$; Figure 1b). The potential for transmission from adults to larvae was higher if *V. culicis* infected mosquitoes early (18.2%, CI 14.55–22.48) than if *E. aedis* infected them late (6.2%, CI 4.19–9.18; $\chi^2 = 29.9$, $df = 1$, $p \ll 0.001$). This was in particular the case for co-infected individuals (aggregated across food treatment), in which early infection by *V. culicis* gave 18.5% (CI 13.52–24.89) potential for transmission from females to larvae and 3.8% when it was the later parasite (CI 1.84–7.60), though this interaction between co-infection and timing was not quite statistically significant ($\chi^2 = 3.65$, $df = 1$, $p = 0.056$). The effect of timing with early infection was stronger when larvae had been reared with high food (19.6%, CI 14.48–25.89 vs. 3.9%, CI 1.91–7.85) than when they had been reared with low food (16%, CI 12.0–22.92 vs. 8.4%, CI 5.25–13.24; $\chi^2 = 4.9$, $df = 1$, 0.027). The other interactions were insignificant ($\chi^2 < 1.35$, $df = 1$, $p > 0.245$).

3.1.3 | *Edhazardia aedis*: Among larvae transmission

Co-infection had no effect on the potential for transmission of *E. aedis* among larvae (i.e. the proportion of mosquitoes that died before emerging and harboured uninucleate spores), either as a main effect ($\chi^2 = 0.1$, $df = 1$, $p = 0.700$) or as interactions with the other factors ($\chi^2 < 0.1$, $df = 1$, $p > 0.833$; Figure 2a). In particular, the order of infection had no effect in co-infected hosts. The potential for transmission among larvae was higher if hosts were infected early (5.2%, CI 3.33–7.92) than if they were infected late (1.4%, CI 0.58–3.14; $\chi^2 = 13.54$, $df = 1$, $p < 0.001$), and higher if they were reared with low food (4.3%, CI 2.67–6.89) than with high food (2.2%, CI 1.11–4.26; $\chi^2 = 7.04$, $df = 1$, $p = 0.008$; Figure 2a). Increasing larval food decreased horizontal transmission more if infection by *E. aedis* was late (from 2.7%, CI 1.17–6.24 to 0%, CI 0–2.03) than if it was early (from 5.9%, CI 3.30–10.170 to 4.4%, CI 2.27–8.52; interaction food × timing: $\chi^2 = 4.8$, $df = 1$, $p = 0.028$).

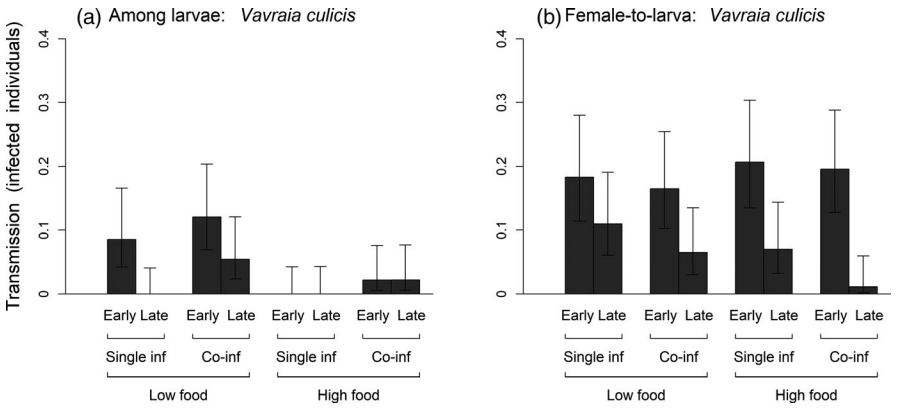

FIGURE 1 Among larvae (a) and female-to-larva (b) transmission potential of *Vavraia culicis*. The bars represent the proportion of infected individuals, the vertical lines show the 95% confidence interval. High and Low labels are, respectively, the high and low food regime, Single inf and Co-inf the single infections and the co-infections, Early and Late the time of infection

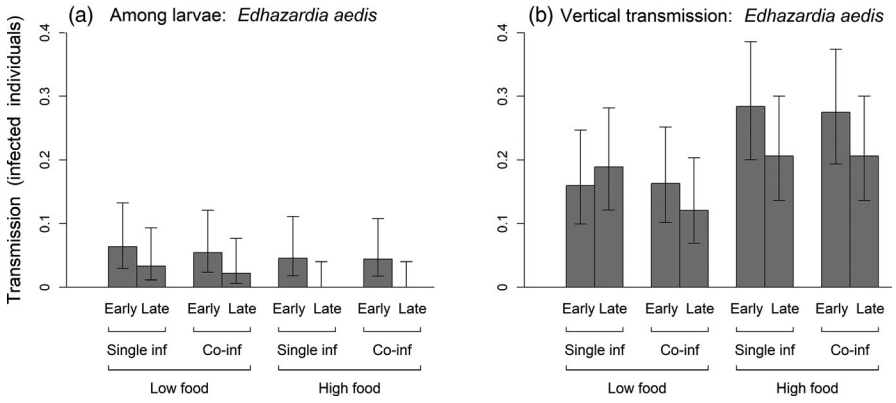

FIGURE 2 Among larvae (a) and vertical (b) transmission potential of *Edhazardia aedis*. The bars represent the proportion of infected individuals, the vertical lines show the 95% confidence interval. High and Low labels are, respectively, the high and low food regime, Single inf and Co-inf the single infections and the co-infections, Early and Late represent the time of infection

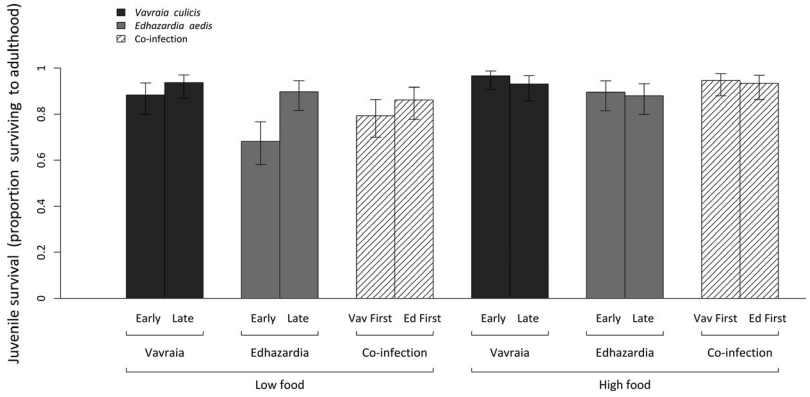

FIGURE 3 Juvenile survival represented as the proportion of individuals surviving to adulthood for *Vavraia culicis* and *Edhazardia aedis*. The dark bars show mosquitoes infected only with *Vavraia*, the light bars those infected only with *Edhazardia*, and the dashed bars the co-infected mosquitoes. The vertical lines show 95% confidence intervals

3.1.4 | *Edhazardia aedis*: Vertical transmission

The only factor that affected the potential of *E. aedis* for vertical transmission (the proportion that survived to become a female and harboured binucleate spores) was food ($\chi^2 = 8.3$, $df = 1$, $p = 0.004$), with low food giving 15.6% (CI 12.29–19.68) and high food giving 24.1% (CI 20.01–28.75) transmission potential (Figure 2b). Neither timing of infection ($\chi^2 = 1.5$, $df = 1$, $p = 0.216$), co-infection ($\chi^2 = 0.6$, $df = 1$, $p = 0.453$) nor any interaction ($\chi^2 < 0.8$, $df = 1$, $p > 0.393$) was linked to the potential of vertical transmission, although there was a slight tendency for later infected mosquitoes to have lower vertical transmission (Figure 2b).

3.2 | Host's life-history traits

3.2.1 | Juvenile survival

Larvae infected only by *V. culicis* were more likely to survive (92.8%, CI 89.68–95.01) than those infected only by *E. aedis* (83.9%, CI 79.76–87.29; mean $\chi^2 = 4.90$, $df = 1$, mean $p = 0.027$), and the survival of co-infected mosquitoes was between these two values (88.4% CI 84.71–91.26; co-infection: mean $\chi^2 = 0.18$, $df = 1$, mean $p = 0.741$: focal parasite × co-infection: mean $\chi^2 = 4.74$, $df = 1$, mean $p = 0.029$). Juveniles with access to high food survived better (92.5%, CI 89.93–94.40) than mosquitoes reared with low food (84.3%, CI 81.03–87.09; mean $\chi^2 = 16.75$, $df = 1$, mean $p \ll 0.001$). The effect of timing tended to be stronger when mosquitoes were reared with low food than with high food (Figure 3), but the interactions of food and timing (mean $\chi^2 = 3.46$, $df = 1$, mean $p = 0.063$) and of food, timing and co-infection (mean $\chi^2 = 3.44$, mean $p = 0.064$) were not quite significant. The other factors and interactions had no effect on juvenile survival (mean $\chi^2 < 1.88$, $df = 1$, mean $p > 0.170$).

3.2.2 | Age at emergence

The age at emergence of females depended on the combination of the focal parasite, the timing of infection and whether hosts were infected by one or two parasites (three-way interaction: mean $\chi^2 = 9.87$, $df = 1$, mean $p = 0.002$; Figure 4; note that for the figure we chose 9 days as a cut-off only to represent long development graphically; the statistics analyses were done with a survival analysis.). If females were co-infected, the age at their emergence depended on the order of the infections. If *Vavraia*-infection was first, they emerged later (57.3% emerged before day 10, CI 45.51–68.4) than if *Vavraia*-infection was second (64.8%, CI 53.5–74.76). If females were infected only with *E. aedis*, time of infection did not influence age at emergence; of the mosquitoes infected early, 61% (CI 48.26–72.42) emerged before or on the median day of emergence (day 9); of those infected late 66.2% (53.5–74.76) did. If, however, females were infected only with *V. culicis*, the time of infection had an impact: they emerged at about the same time as *Edhazardia*-infected ones if their infection was early (60.5%, CI

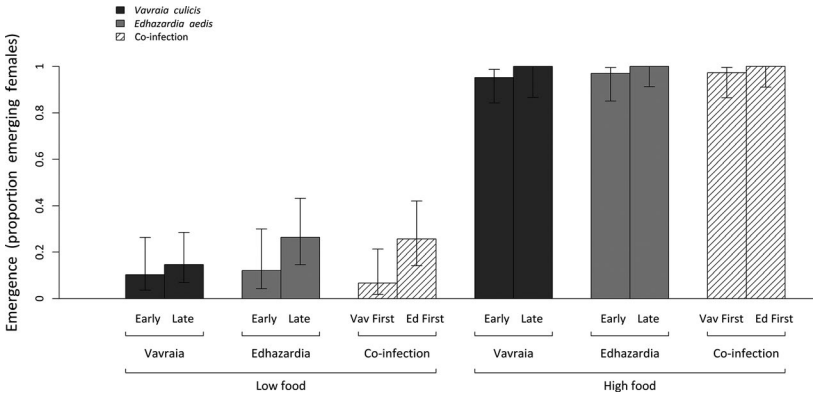

FIGURE 4 Age at emergence of female mosquitoes. The graph represents the proportion of females emerging up to (and including) day 9 (the median age at emergence) for *Vavraia culicis* and *Edhazardia aedis*. The dark bars show mosquitoes infected only with *Vavraia*, the light bars those infected only with *Edhazardia*, and the dashed bars the co-infected mosquitoes. The vertical lines show the 95% confidence intervals

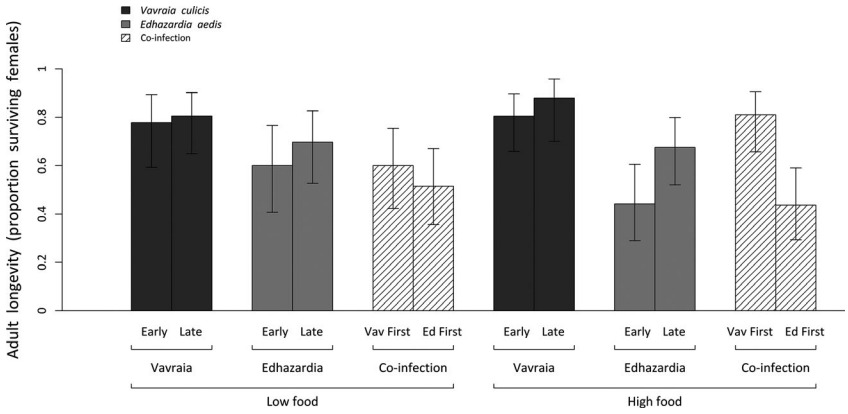

FIGURE 5 Adult longevity. Proportion of adult females surviving up to day 10 post emergence for *Vavraia culicis* and *Edhazardia aedis*. The dark bars show mosquitoes infected only with *Vavraia*, the light bars those infected only with *Edhazardia* and the dashed bars the co-infected mosquitoes. The vertical lines show the 95% confidence intervals

48.93–71.10), but emerged later if their infection was late (46.9% CI 35.43–58.84). Food had the largest impact on age at emergence (mean $\chi^2 = 318.3$, $df = 1$, mean $p \ll 0.001$), with 16.4% (CI 11.1–23.6) of low food-nourished females and 98.1% (CI 94.4–99.4) of the high food-nourished females emerging before day 10. Food also has a small effect on how the combination of focal parasite and timing of infection affected age at emergence (three-way interaction: mean $\chi^2 = 4.43$, $df = 1$, mean $p = 0.035$; Figure 4), but food had no effect on the impact of co-infection (all interactions, mean $\chi^2 < 1.76$, $df = 1$, mean $p > 0.184$).

3.2.3 | Longevity of females

Co-infected mosquitoes lived less long (62% lived more than 10 days, CI 54.19–69.97) than singly infected mosquitoes (76.1%, CI 65.34–78.00; survival analysis: mean $\chi^2 = 7.37$, $df = 1$, mean $p = 0.007$; Figure 5). Note that we chose 10 days as a cut-off only to represent longevity in the figure; the statistics were done with a survival analysis. Ten days is the age at which the mosquitoes with the latest emergence were censored. The effect of co-infection was stronger in comparison with those infected only by *V. culicis* (84.62%, CI 77.43–89.81) than in comparison with those infected by *E.* aedis (68.6% lived more than 10 days, CI 0.42–75.78; focal parasite: mean $\chi^2 = 14.58$, $df = 1$, mean $p \ll 0.001$, co-infection × focal parasite: mean $\chi^2 = 13.19$, $df = 1$, mean $p = 0.003$). The shorter longevity after infection by *E. aedis* was mainly apparent if infection was early (54.1% lived more than 10 days, CI 41.72–65.99) than if it was late (80.3%, CI 69.96–87.66), whereas timing of infection had no influence on longevity if mosquitoes were infected by *V. culicis* (early infection: 82.6%, CI 72.02–89.76; late infection: 86.9%, CI 76.20–93.20; survival analysis: focal parasite × timing: mean $\chi^2 = 13.17$, $df = 1$, mean $p = 0.003$) Food had no effect on longevity, either as a main effect or in interactions (survival analysis: mean $\chi^2 < 1.93$, $df = 1$, mean $p > 0.165$).

4 | DISCUSSION

In this study, we investigated how transmission and virulence of two microsporidian parasites and their effect on the life-history traits of the host were affected by co-infection, order and time of infection and the food available to the host. We discuss the main results in function of our three core predictions.

4.1 | Co-infection

As expected, if *V. culicis* encountered a co-infecting parasite, it increased its potential for horizontal transmission, in particular at low food conditions. This response to co-infection may give *V. culicis* the chance to produce spores and kill its host before its competitor, and thus give it a competitive advantage over *E. aedis* in the juvenile stage of the host. The shift is consistent with competition among parasites for shared resources (Fellous & Koella, 2009;

Mideo, 2009). Alternatively, parasites may be less prudent in co-infections (Frank, 1996; van Baalen & Sabelis, 1995) and maximize host exploitation, the classical view of the tragedy of the commons (Frank, 1996; Levin & Bull, 1994).

However, contrary to our expectation, *E. aedis* increased neither its potential for vertical transmission nor its horizontal transmission if its host was co-infected with *V. culicis*. There are several possible reasons for the lack of a response to co-infection, one of which could be the specificity of the parasite. First, the development of *E. aedis* relies on several specific pathways of protein modification and trafficking (Desjardins et al., 2015), and the parasite moves between several tissues as its host develops, until it ends up in the females' ovaries for vertical transmission. *V. culicis*, in contrast, focuses on replication and basic metabolic functions, and it stays in the initially infected tissue until adulthood (Desjardins et al., 2015). The presence of *V. culicis* will not interfere much with the molecular machinery and progression of infection of *E. aedis*. Second, since *E. aedis* strongly stimulates the immune response (Desjardins et al., 2015), it must have evolved mechanisms to avoid immune responses, including those stimulated by *V. culicis*, for no effective responses are known. In contrast, since *V. culicis* relies on a strategy of stealth, stimulating only slightly the immune response (Desjardins et al., 2015), the processes activated by *E. aedis* are likely to impact *V. culicis*, so that it must respond by changing its development. Third, *E.* aedis relies on two spore types for horizontal and vertical transmission, and the parasite's life cycle must alternate between the production of these spore types. This constrains the ability of the parasite to respond to slight changes of the host's mortality induced by a co-infecting parasite (although it can respond to changes in the host's duration of development [see below]).

4.2 | Order of co-infection

In co-infected hosts, the order of infection influenced the success of *V. culicis*, but not that of *E. aedis*. If *V. culicis* was the first parasite in co-infected hosts, it had more transmission among larvae than if it was the second infection. Since co-infection decreased larval survival, this may be seen as a response to increase transmission among larvae (Hoverman et al., 2013; Natsopoulou et al., 2015). However, although co-infected mosquitoes survived less long than *Vavraia*-infected ones, *V. culicis* also had more transmission from females to larvae if it infected the host first. The observed response is thus not due to change of the allocation to the two transmission strategies, but may reflect a general increase in allocation to transmission in an attempt to leave the host before it is killed by the co-infection.

Changes in the outcome of co-infection due to the order of arrival of different parasite species have also been found in other systems (Clay, Dhir, et al., 2019; Hood, 2003; Hoverman et al., 2013; Marchetto & Power, 2018), with the effects scaling up to affect host and parasite population dynamics and the spread of disease in some cases (Clay, Dhir, et al., 2019; Marchetto & Power, 2018). For example, when infecting first, the trematode parasite *Echinostoma trivolvis* reduced the infection success of the second arrival trematode *Ribeiroia ondatrae* (Hoverman et al., 2013). Similarly, the genotypes of the fungus *Microbotryum violaceum* infecting first had an advantage over the second (Hood, 2003). When the bacterial *Pasteuria ramosa* and the fungal parasite *Metschnikowia bicuspidate* infect the host *Daphnia dentifera*, the effect of co-infection differed between the parasites (Clay, Dhir, et al., 2019), as it did in our study. Although the production of bacterial spores (used as a proxy for transmission) was not affected by the order of infection, the co-infecting fungal parasite produced more spores if it infected the host after the bacteria. The same fungal parasite also performed better, if it infected a close related host species, *Daphnia galeata*, after the co-infecting protozoan parasite *Caullerya mesnili* (Lohr, Yin, & Wolinska, 2010). In some co-infections, the order of infection affects neither parasite, as is the case to two viruses infecting barley (Marchetto & Power, 2018). Such a specificity of results is likely the result of the detailed interactions between the different species, and it emphasizes the need to investigate specific cases and both sequences of co-infection of the two parasites when considering sequential co-infection.

In our case, the difference between *V. culicis* and *E. aedis* may, again, be due to details of the two parasites' development. It may also reflect differences in the selection pressures, for larvae survived better if they were co-infected than if they were infected only with *E. aedis*, and the effect of co-infection on longevity was less for *Edhazardia*-infected than for *Vavraia*-infected mosquitoes. Thus, there is little pressure for *E. aedis* to change its strategy, whether it encountered a co-infecting parasite early or late.

4.3 | Food and timing of single infections

While the effect of food matched our predictions for transmission, with less food giving more horizontal transmission of both parasites, the effect of the timing of infection did not, for early infection did not shift the allocation towards horizontal transmission. While early infection of either parasite increased its horizontal transmission, it had no effect on vertical transmission of *E. aedis*, and increased vertical transmission of *V. culicis*.

While the effects of the environment (the amount of food and the timing of infection) partially matched our predictions concerning transmission, the outcomes for adult longevity were unexpected. First, although the amount of food available to larvae generally affects the longevity of uninfected mosquitoes (e.g. Barreaux, Stone, Barreaux, & Koella, 2018), it had no impact on the longevity of the infected mosquitoes of our experiment. A similar difference between infected and uninfected mosquitoes was seen in Bedhomme et al. (2004). Low food, however, decreased juvenile survival and delayed emergence, which increased horizontal transmission potential for both parasites, as seen in other studies (Agnew & Koella, 1999; Zilio et al., 2018). Timing of infection affected longevity only if the mosquitoes were infected by *E. aedis*, with earlier infection shortening the life span of adults.

This reflects that the transmission strategy of *E. aedis* is tightly linked with the duration of infection and host development (Zilio et al., 2018). A longer period of infection within the juveniles enables the parasite to produce its horizontally transmitted spores (Agnew & Koella, 1999). A shorter period forces the parasite to transmit vertically, so that it has greater interest in its host's longevity (Frank, 1996). Indeed, low virulence has observed in several vertically transmitted parasites (Ferrari, Darby, Daniell, Godfray, & Douglas, 2004; Hedges et al., 2008; Jaenike, Unckless, Cockburn, Boelio, & Perlman, 2010). The timing of infection of *V. culicis*, in contrast, had no impact on longevity. Thus, investment in transmission affected longevity similarly, whether transmission was among larvae or from females to larvae. This may reflect that *V. culicis* is not constrained by different spore types for its two transmission routes, so that the impact of its investment in transmissible spores is independent of the life-stage of its host.

The difference of only 2 days for the parasite to develop, and consequently the infection of a host with a different age, thus had a large impact on shaping infection strategies and virulence evolution in either single or co-infection, as seen for other parasites (Clerc, Ebert, & Hall, 2015; Gipson & Hall, 2018; Izhar, Routtu, & Ben-Ami, 2015; Jager & Schorring, 2006). That early infection increases transmission can be expected to lead to an epidemiological feedback, for more intense transmission leads to earlier infection.

5 | CONCLUSIONS

Our experiment highlights that interactions among parasites within their host, and in particular the order of infections, play an important role in determining the outcome of infections for the parasite and for the host. It also shows that it is difficult to predict the outcome of co-infections from the outcome of single infections and from simple evolutionary considerations. Rather, the competitive interactions between parasites appear to be influenced by subtle details of how each parasite interacts with its host and by the constraints of its development. Thus, although single infections of *V. culicis* and *E. aedis* responded similarly to environment variation, *V. culicis* was affected more by co-infection with *E. aedis* than was *E. aedis* by co-infection with *V. culicis*. It is tempting to speculate that this asymmetry is linked to the complexity of the parasites' development: *V. culicis* has one type of spore and develops mainly in the fat body, while *E. aedis* has two spore types dedicated to horizontal and vertical transmission and develops in several tissues and organs.

More generally, since co-infections are common, and since we cannot yet understand many of their outcomes for the parasite, for the host and thus for epidemiology, we need more effort to find general patterns and to obtain a better comprehension of host–parasite evolution.

ACKNOWLEDGEMENTS

We thank Oliver Kaltz and Daniel Croll for helpful discussions, the MSc students of 'Experimental parasitology' for their help in the laboratory and the reviewers for their comments and efforts towards improving this manuscript.

AUTHORS' CONTRIBUTIONS

Both authors contributed substantially to the conception of the ideas, the design of the experiment, the analysis of the data and to the writing of the manuscript. Both gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.3bk3j9kgm (Zilio & Koella, 2020).

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How to cite this article: Zilio G, Koella JC. Sequential co-infections drive parasite competition and the outcome of infection. *J Anim Ecol*. 2020;89:2367–2377. https://doi.org/10.1111/1365-2656.13302