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The consequences of co-infections for parasite transmission in the mosquito *Aedes aegypti*

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Summary

1. Co-infections may modify parasite transmission opportunities directly as a consequence of interactions in the within-host environment, but also indirectly through changes in host life history. Furthermore, host and parasite traits are sensitive to the abiotic environment with variable consequences for parasite transmission in co-infections.

2. We investigate how co-infection of the mosquito *Aedes aegypti* with two microsporidian parasites (*Vavraia culicis* and *Edhazardia aedis*) at two levels of larval food availability affects parasite transmission directly, and indirectly through effects on host traits.

3. In a laboratory infection experiment, we compared how co-infection, at low and high larval food availability, affected the probability of infection, within-host growth and the transmission potential of each parasite, compared to single infections. Horizontal transmission was deemed possible for both parasites when infected hosts died harbouring horizontally transmitting spores. Vertical transmission was judged possible for *E. aedis* when infected females emerged as adults. We also compared the total input number of spores used to seed infections with output number, in single and co-infections for each parasite.

4. The effects of co-infection on parasite fitness were complex, especially for *V. culicis*. In low larval food conditions, co-infection increased the chances of mosquitoes dying as larvae or pupae, thus increasing opportunities for *V. culicis'* horizontal transmission. However, co-infection reduced larval longevity and hence time available for *V. culicis* spore production. Overall, there was a negative net effect of co-infection on *V. culicis*, whereby the number of spores produced was less than the number used to seed infection. Co-infections also negatively affected horizontal transmission of the more virulent parasite, *E. aedis,* through reduced longevity of pre-adult hosts. However, its potential transmission suffered less relative to *V. culicis*.

5. Our results show that co-infection can negatively affect parasite transmission opportunities, both directly as well as indirectly via effects on host life history. We also find that transmission is contingent on the combined effect of the abiotic environment.

Key-words: *Aedes aegypti*, co-infection, *Edhazardia aedis*, horizontal transmission, *Vavraia culicis*, vertical transmission

Introduction

Organisms are commonly infected with multiple parasite strains or species (Petney & Andrews 1998; Cox 2001). This is important for parasite evolution and epidemiology as co-infection can modify host and parasite life history differently to single infections. Indeed, consistent with prevailing theory (reviewed in Mideo 2009; Alizon, de Roode & Michalakis 2013), empirical investigations reveal that competition between different strains of the same parasite is often associated with enhanced parasite growth and virulence (defined as parasite induced harm to the host) (Bell *et al.* 2006; Ben-Ami, Mouton & Ebert 2008; de Roode *et al.* 2005), but see Gower & Webster (2005). In contrast, the outcomes for host and parasite life history are more variable when co-infections comprise different

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parasite species (e.g. Haine, Boucansaud & Rigaud 2005; Hughes *et al*. 2004; Solter *et al*. 2002). This may be attributed to parasite species having very different and possibly conflicting life-history strategies, such as, the timing or mode of transmission.

Co-infecting parasites with contrasting transmission modes are likely to impose strong selection on each other, as there is potential for a clear conflict of interest for host use. Vertically transmitted parasites are typically selected to minimize host harm so that host reproduction and transmission are maximized (Fine 1975). Conversely, horizontally transmitted parasites, unaffected by host reproduction, are often more virulent as higher within-host growth, and thus potentially virulence, is often associated with increased transmission (Alizon *et al*. 2009). Studies show that vertically transmitted parasites can protect against subsequent infection with horizontally transmitted parasites (Jones, White & Boots 2011; Rigaud & Haine 2005) and impose selection for their increased virulence (Jones, White & Boots 2010). However, these studies are restricted to co-infections comprising parasites with strictly horizontal or vertical transmission and not parasites capable of both modes of transmission. A recent investigation highlights that outcomes for co-infection between a strictly horizontally transmitted parasite and a parasite with both modes of transmission depend on the infection route of the latter (Ben-Ami, Rigaud & Ebert 2011). Considering the large number of parasites capable of both horizontal and vertical transmission, further study is required to understand the epidemiological and evolutionary consequences of competition between parasites with contrasting transmission modes (Ebert 2013).

Environmental variation is another factor that can impact parasite transmission directly, or indirectly via effects on host life history (Lazzaro & Little 2009; Wolinska & King 2009). Food limitation has the potential to reduce parasite transmission by constraining host resources available for parasite growth (Ferguson & Read 2002; Bedhomme *et al*. 2004; Lambrechts *et al*. 2006; Takken *et al*. 2013; Vale, Choisy & Little 2013). Alternatively, food limitation can benefit parasites if host development is arrested at a life stage favouring their transmission (Agnew & Koella 1999; Bedhomme *et al*. 2004) or reduces the host's ability to mount an efficient immune response. Thus, food availability has the potential to impact parasite transmission in co-infections if interactions are mediated via host resources or immune factors, or if effects on host life-history impact conflicts regarding the timing or routes of transmission. For example, if co-infecting parasites compete for limited host resources, it is probable that variation in food availability will influence transmission in co-infections. Consistent with this, a recent study found that the effects of parasite dose on competing parasite species were maximal under limiting host food (Fellous & Koella 2009).

Here, we investigate the effects of co-infection of the mosquito *Aedes aegypti* with two microsporidian parasites in conditions of high or low larval food availability. The two parasites, *Vavraia culicis* and *Edhazardia aedis*, both use uninucleate spores for horizontal transmission; this is the only mode of transmission available to *V. culicis*, whereas *E. aedis* also produces binucleate spores used in vertical transmission. Horizontal transmission of *V. culicis* occurs primarily in the aquatic environment when larvae ingest spores released from the cadavers of infected larvae or pupae. Horizontal transmission of *E. aedis* occurs in the same way, while vertical transmission is transovarial from mother to offspring. Low larval food availability influences *V. culicis* both directly through reductions in its own growth rate and indirectly through effects on host life history, increasing the proportion of mosquitoes dying as larvae or pupae in water where opportunities for horizontal transmission are greater (Bedhomme *et al*. 2004). In contrast, the effects of food stress on *E. aedis* life history are indirect, mediated by effects on mosquito life history, with horizontal transmission arising when infected mosquitoes remain as larvae or pupae, and vertical transmission when they emerge as adults (Agnew & Koella 1999). These studies highlight that the fitness of both parasites is tightly linked to their host's life history.

We examine the direct impact of co-infection and food availability on the transmission potential of each parasite. We investigate their effects on different components of the transmission potential: (i) the probability that hosts become infected; (ii) the effects on host life-history traits which have indirect consequences for parasite transmission, namely the proportion of mosquitoes reaching adulthood and the longevity of larvae that do not. Adult emergence has obvious fitness consequences for the mosquito host, but also for both parasites as it determines the likelihood of horizontal transmission following release of spores from cadavers in water. The longevity of larvae or pupae provides an indication of parasite virulence, but also impacts the fitness of both parasites through time available for spore production; (iii) the probability that hosts harbour transmissible spores when they die in each environment (aquatic or aerial); and (iv) the number of transmissible spores they carry. We then integrate all these variables into one synthetic variable, the total number of transmissible spores produced by each parasite in each treatment, to infer the overall effect on each parasite.

Co-infection might impact parasite fitness directly due to the presence of a competitor in the within-host environment, but also via effects on host life history. Parasite growth and therefore potential transmission might be reduced if co-infecting parasites interact via shared host resources or immune responses (Mideo 2009; Alizon, de Roode & Michalakis 2013). Indeed, another study reported reduced *V. culicis* growth in co-infections (Fellous & Koella 2009). Co-infections may be more virulent than corresponding single infections, reducing the probability that a mosquito emerges as an adult, therefore increasing opportunities for horizontal transmission of

these parasites. Food availability was expected to impact parasite fitness independently of co-infection in two contrasting ways: we expected low food conditions (i) to increase potential horizontal transmission through an increase in the probability that hosts would die in the aquatic environment and (ii) to decrease potential horizontal transmission through a decrease in the production of spores, relative to high food conditions. Indeed, we previously observed that increased food availability leads to higher *V. culicis* spore production (Bedhomme *et al.* 2004). We did not have a prediction on the overall effect combining these two components, or for their interaction with co-infections: we expected co-infections to increase the magnitude of both effects, but we could not *a priori* predict the net outcome.

Materials and methods

EXPERIMENTAL SYSTEM

Mosquitoes

Aedes aegypti occurs throughout the tropics and subtropics where it is the principle vector of dengue and yellow fever. Its larvae are found in a variety of natural and artificial small, clean freshwater bodies. Larval development varies from 6 to more than 30 days depending on environmental conditions (Southwood *et al.* 1972). The *A. aegypti* population used in this study derives from a large number of eggs collected in Tingua, Brazil, by Ricardo Lorenço de Oliveira of the Instituto Oswaldo Cruz (Rio de Janeiro, Brazil). They have since been maintained in standardized laboratory conditions with more than 3000 reproductive adults in each generation. The population was kept at 25 °C (±3 °C), 75% (±5%) humidity with a 12 h : 12 h light : dark photoperiod. At the time of this experiment, the population had been maintained for 20 generations in our laboratory.

Microsporidia

The two species of microsporidia used in this experiment, *Vavraia culicis* and *Edhazardia aedis*, are both natural parasites of *A. aegypti*. *V. culicis* has been reported from several genera of mosquitoes (Weiser & Coluzzi 1972), while *E. aedis* has only been reported from *A. aegypti* (Andreadis 1994). In both cases, horizontal transmission occurs when larvae ingest spores with their food. Conditions in the mid-gut stimulate the germination of spores, resulting in the expulsion of a long hollow polar tube thought capable of traversing the peritrophic matrix and piercing host cells lining the gut so initiating infection.

Once in the cytoplasm, *V. culicis* undergoes a series of developmental stages that culminate in the production of a single type of uninucleate spore. These spores are infectious to neighbouring cells within the host or to other larvae once released into an aquatic environment following host death. The costs experienced by the host and the number of spores produced by the parasite increase with time from infection and depend on the amount of food available to the host (Bedhomme *et al.* 2004). This parasite is only transmitted horizontally, mainly when infected larvae or pupae die in water (Kelly, Anthony & Dillard 1981). Hence, the persistence of *V. culicis* in a site largely depends on the release of spores from infected hosts dying as larvae or pupae, as opposed to those emerging and dispersing as adults. Spores in adults are the only ones contributing to the colonization of new sites. However, adultborne spores can contribute to horizontal transmission upon returning to the aquatic environment. As the spores of microsporidian species infecting aquatic hosts are sensitive to desiccation (Becnel & Andreadis 1999), horizontal transmission from infected adults is only likely if they die directly on or in larval breeding sites.

Edhazardia aedis has a more complicated life cycle involving both horizontal and vertical transmission. Larvae ingest uninucleate spores with their food, and these germinate to infect cells lining the host mid-gut. An initial sequence of development leads to the production of binucleate spores. These spores are responsible for the transmission of infection to other cells within the same host (auto-infection). A second type of binucleate spore is then produced within infected cells and is responsible for vertical (transovarial) transmission (Johnson, Becnel & Undeen 1997). Further development occurs in the fat body of vertically infected offspring and includes the production of uninucleate spores, the accumulation of which is associated with larval and pupal mortality and the release of spores into the aquatic environment. The life cycle of *E. aedis* as described above includes a sequence of horizontal and then vertical transmission in sequential mosquito generations. Repeated sequences of either horizontal or vertical transmission may also occur if horizontally infected larvae fail to emerge as adults, or if vertically infected females survive to adulthood (Becnel *et al.* 1989; Agnew & Koella 1999).

The *V. culicis* and *E. aedis* materials used in this experiment were originally provided by Dr J.J. Becnel of the USDA/ARS (Gainesville, Florida, USA), with *V. culicis* being the Florida isolate *V. culicis floridensis* (Vávra & Becnel 2007). Throughout the paper, we refer to these isolates as '*V. culicis*' and '*E. aedis*' for brevity. However, we recognize that our results could be specific to the isolates we used; thus, generalization to other populations of the same species should be done with caution.

EXPERIMENTAL PROCEDURE

General outline

The goal of the experiment was to investigate the effects of co-infection on the transmission of both parasites, and how food availability mediates these effects. After exposing groups of newly hatched larvae to spores of either parasite species alone or in combination, along with unexposed control individuals, we reared individual larvae in one of two larval food conditions: high or low (Fig. S1, Supporting information). The high food conditions allow uninfected larvae to develop rapidly with most emerging as adults, whereas the low food condition reflects field conditions where larval growth and emergence are often constrained by limiting food availability (Arrivillaga & Barrera 2004). During the experiment, we recorded if and when mosquitoes pupated or emerged on a daily basis, and when they died, along with the sex of emerging adults. The number of spores produced by either parasite was quantified for infected individuals. Spore counts and the stage at which the host died were used to infer the transmission potential of each parasite from each treatment. Infection was verified *a posteriori,* and only individuals whose infection status matched their exposition status were used in the analyses

(unless exposed uninfected individuals were relevant, e.g. for looking at effects on infection rates).

Detailed procedures

Larvae were hatched by placing eggs in a cup containing 150 mL of mineral water in a vacuum chamber. Groups of 60 larvae were transferred to Petri dishes (55 mm diameter) containing mineral water (Eau de Source, Carrefour, France). Two Petri dishes per treatment were randomly assigned to four infection treatments: (1) *V. culicis* alone, (2) *E. aedis* alone, (3) *V. culicis* and *E. aedis* together and (4) controls. Petri dishes assigned to infection treatments (1) and (3) received *V. culicis* spores (2×10^4 spores per larva) suspended in 1 ml of mineral water, and Petri dishes assigned to treatments (2) and (3) received *E. aedis* uninucleate spores (100 spores per larva) suspended in 1 ml of mineral water. These spore densities were chosen because they lead to *c.* 90% infection rates. Petri dishes assigned to treatments (1) and (2) received 1 ml of mineral water, whereas Petri dishes assigned to treatment (4) received 2×1 ml of mineral water to equalize water volumes across treatments. All Petri dishes received 3·6 mg of Tetramin fish food. After 24 h (Day 1), larvae from each Petri dish within each treatment were combined and rinsed. Larvae were then randomly assigned to individual tubes containing 4 ml of mineral water. Each larva was reared in its own tube arranged in racks containing 4 rows of 10 tubes, each row containing a single treatment. The eight treatment conditions were assigned to pairs of racks, with one row of each infection condition in each rack and two rows receiving the high food, and two the low food treatment. The order of rows within individual racks was randomized. Tubes assigned to the high food treatment received 2 mg of Tetramin fish food dissolved in 1 ml of mineral water, while tubes assigned to the low food treatment received 1·4 mg of Tetramin fish food dissolved in 1 ml of mineral water. Our target sample size was 80 larvae per treatment. The experiment was maintained in a room at 25 °C (±1 °C), 75% (±5%) humidity with a 12 L : 12 D photoperiod, and the position of racks was rotated daily.

Tubes were checked every 24 h, and the ages of pupation, emergence and death were recorded. In the event of pupation, a foam bung was placed in the tube to prevent emerging mosquitoes from escaping and adult sex recorded. Following death, each individual was transferred to a 1·5-ml Eppendorf tube and frozen at −20 °C. All dead adult mosquitoes were dried in an oven for 12 h at 65 °C and weighed using a Mettler MX5 microbalance (Mettler-Toledo GmbH, Greifensee, Switzerland).

To confirm infection, all mosquitoes exposed to parasites were crushed individually in their Eppendorf tubes in 0·5 ml of de-ionized water. *V. culicis* infection was mostly confirmed using microscopy by placing 8 µl of this solution on a Neubauer haemocytometer where spores were identified and counted. *E. aedis* infection was confirmed and quantified in the same way when uninucleate spores were present. Although uninucleate spores are easily distinguishable from binucleate spores with light microscopy, the different types of binucleate spores are only distinguishable with electron microscopy. As such, binucleate spore observations were recorded under a single category. Infection was confirmed using polymerase chain reaction (PCR) in instances where spores could not be identified under the microscope (methods described in Duncan *et al.* 2012). We identified 83% of *V. culicis* infections using microscopy. Conversely, for *E. aedis*, we observed spores in only 54% (27% uninucleate spores only, 17% binucleate spores only, 10% both types of spores) of infected mosquitoes and confirmed the remaining 46% infections using PCR.

STATISTICAL ANALYSIS

Probability of infection

We used nominal logistic regressions to investigate how parasite treatment and larval food availability affected the probability of infection with each parasite. This analysis included all mosquitoes exposed to parasites that were checked for infection using either microscopy or PCR.

Qualitative estimates of parasite transmission from larvae and pupae

The proportion of mosquitoes dying as larvae or pupae greatly increases the probability of horizontal transmission from infected hosts. Indeed, horizontal transmission of both parasites occurs when uninucleate spores are released from the cadavers of infected hosts in water. Horizontal transmission from infected larvae and pupae is *a priori* possible, for each parasite, from individuals confirmed to harbour uninucleate spores. Mosquitoes defined as not contributing to horizontal transmission from larvae or pupae included those that were uninfected or infected without harbouring uninucleate spores and all adults. Infected larvae and pupae not checked for the presence of spores were excluded from analyses. The impact of parasite treatment and larval food on (1) the proportion of mosquitoes dying as larvae or pupae and (2) the proportion of larvae and pupae affording horizontal transmission, both important measurements of parasite fitness, were analysed using nominal logistic regression.

Mosquitoes that died before day 5 were excluded from analyses, as it was not possible to confirm *V. culicis* infection by either microscope or PCR (see Table S1, Supporting information). Consequently, we tested whether the qualitative estimates of transmission success described above were influenced by the inclusion of these individuals in the category of non-transmitting mosquitoes. Their inclusion in models only modified analyses investigating *V. culicis'* horizontal transmission. As such, we only report the results of this model.

Quantitative estimates of parasite spore production in infected larvae and pupae

We used Weibull survival analysis to investigate the effects of parasite treatment on larval and pupal longevity, a reflection of the time available for parasite growth within infected hosts. Analyses of covariance (ANCOVA) were used to investigate whether the log number of spores produced for each parasite was affected by co-infection and food availability. As the number of spores increases with time, mosquito longevity was included in models as a covariate. One larva, confirmed to be an outlier using outlier analysis, was excluded from the analysis for *V. culicis*.

These analyses were restricted to aquatic stage mosquitoes in the low food treatment only. For the survival and *V. culicis* spore production analyses, this was because all, and all but 4, mosquitoes emerged as adults in the high food control and single

V. culicis groups, respectively. Few individuals infected with *E. aedis* died as larvae or pupae in the high food treatments and harboured uninucleate spores ($n < 5$), precluding an ANCOVA of these data.

We also compared how the number of spores used to initiate each parasite's infection related to the number of spores that would be available for horizontal transmission from individuals dying in water in the different treatments. The latter integrates all host and parasite life-history traits relevant to the production of uninucleate spores used in horizontal transmission: probability of infection, probability that hosts die in water harbouring uninucleate spores and the number of these spores. It can thus be viewed as an integrative quantitative measure of the potential for horizontal transmission of each parasite under our experimental conditions. This would be equivalent to R0, if instead of summing spore production across individuals we had used average spore production weighted by the probabilities of infection and dying in water harbouring uninucleate spores.

In order to obtain 95% confidence intervals for the total number of spores produced by each parasite species in each treatment, we bootstrapped across all individuals in each treatment, which had been checked for the presence of spores. Thus, not only the total number of spores varied across bootstraps but also the infection rate, depending on the number of exposed-but-uninfected individuals drawn in each replicate. The confidence intervals were obtained from 1000 bootstraps in each case. We only show this for each parasite in the low food conditions due to the limited amount of data available from larvae in the high food conditions.

Qualitative estimates of parasite transmission success from adults

The horizontal transmission of both parasites, and the vertical transmission of *E. aedis*, is in principle possible from infected adult mosquitoes. Nominal logistic regressions were used to investigate how parasite treatment and larval food availability affected qualitative estimates of horizontal transmission potentially afforded, for both parasites from infected adults, and vertical transmission of *E. aedis*. Horizontal transmission from infected adults was defined as only being possible, for each parasite, when they were confirmed to harbour uninucleate spores. The remaining individuals, including infected adults not harbouring uninucleate spores, were classed as not potentially offering horizontal transmission. Handling errors meant some infected adults (n = 20) were not checked for the presence of spores; these individuals were excluded from these analyses. Potential vertical transmission of *E. aedis* was considered possible from all adult female mosquitoes infected by this parasite. Infected individuals dying as larvae or pupae and emerging infected male mosquitoes were classed as not offering potential vertical transmission to *E. aedis*.

The probability of horizontal transmission for either parasite from infected adults is difficult to estimate as it relies on hosts dying on water bodies containing susceptible larvae. As such, we interpret these results with caution, as a qualitative indication of the direction of an effect.

Unless specified, analyses only include individuals that were positive for infection with the parasites to which they were exposed. All analyses were done using R 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria) or JMP 7 (SAS Institute Inc., Cary, NC, USA).

Results

This experiment explored how the potential transmission of *V. culicis* and *E. aedis* differed in co-infections as compared to corresponding single infections and how these responses were modulated by larval food availability. Below we report how the single and co-infection parasite treatments affected the infection success and the type and amount of transmission potentially available to each parasite. We first outline how our treatments impact horizontal transmission of each parasite from hosts dying as larvae or pupae through effects on both host and parasite life history. The potential horizontal transmission of each parasite from hosts emerging and dying as adults is also considered (See Tables S2 and S3, Supporting information).

PROBABILITY OF INFECTION

The probability of infection for *V. culicis* was influenced by the presence of *E. aedis* ($\chi^2_1 = 4{\cdot}88$, $P = 0{\cdot}034$), and a significant interaction showed the direction of the effect to be influenced by larval food availability ($\chi^2_1 = 11{\cdot}55$, $P = 0{\cdot}0007$). The probability of infection for *V. culicis* was highest in hosts with single infections in the high food treatment [probability of infection and 95% confidence interval: 0·945 (0·867–0·978)] and lowest in the co-infection treatment receiving high larval food [0·69 (0·54–0·809)]. The respective values for the low food treatments were 0·776 (0·671–0·855) for single infections and 0·843 (0·720–0·918) for co-infections. In contrast, the probability of infection for *E. aedis* ranged between *c*. 85 and *c*. 90% and was not affected by the presence of *V. culicis* ($\chi^2_1 = 0{\cdot}07$, $P = 0{\cdot}79$), larval food treatment ($\chi^2_1 = 0{\cdot}05$, $P = 0{\cdot}82$) or their interaction ($\chi^2_1 = 0{\cdot}18$, $P = 0{\cdot}67$).

QUALITATIVE ESTIMATES OF PARASITE TRANSMISSION SUCCESS FROM LARVAE AND PUPAE

Probability of dying in water

Whether mosquitoes die in water before they emerge is an important determinant of the type of transmission available to each parasite (Fig. 1). In general, hosts were more likely to die in water in the low food treatments ($\chi^2_1 = 50{\cdot}34$, $P < 0{\cdot}001$). There was also an effect of the infection treatments ($\chi^2_3 = 138{\cdot}75$, $P < 0{\cdot}001$), such that mosquitoes in the single *E. aedis* and co-infection treatments died in water more often than those from the control treatment or with single *V. culicis* infections. An interaction between food and infection treatments ($\chi^2_3 = 10{\cdot}34$, $P = 0{\cdot}016$) found that the high larval food treatment significantly decreased the proba-

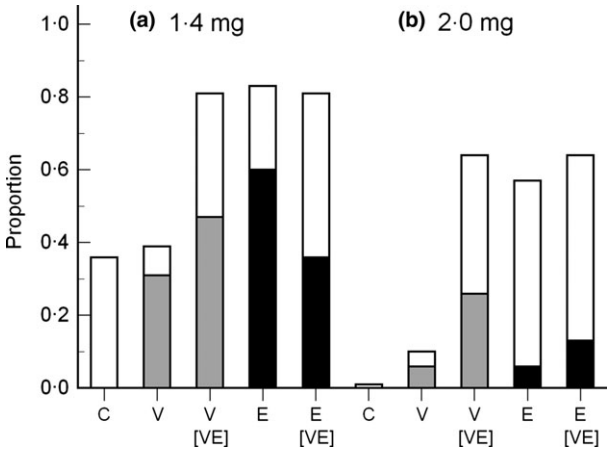

Fig. 1. The proportion of mosquitoes dying as larvae and pupae in the aquatic environment, and of those the proportion affording the potential horizontal transmission of *V. culicis* (grey shading) and *E. aedis* (black shading), at (a) low and (b) high larval food availability in the different parasite treatments. The different parasite treatments are denoted as C = uninfected control, V = *Vavraia culicis* and E = *E. aedis*, and VE indicates the co-infection treatment.

bility of dying in water for uninfected mosquitoes and those with single infections, but not for those with co-infections (Fig. 1).

Qualitative estimates of potential V. culicis *transmission from larvae and pupae*

The potential for horizontal transmission success of *V. culicis* from infected larvae and pupae was significantly higher from hosts in the low food treatment ($\chi^2_1 = 16{\cdot}36$, $P < 0{\cdot}001$) and from those infected with both parasites ($\chi^2_1 = 12{\cdot}08$, $P = 0{\cdot}002$) (Fig. 1). There was no interaction between parasite treatment and food availability for potential horizontal transmission from infected larvae or pupae ($\chi^2_1 = 1{\cdot}60$, $P = 0{\cdot}21$). If individuals whose infection status was unknown at the time of their death (i.e. before day 5) were added to the category of non-transmitting hosts in this analysis, the effect of infection treatment became non-significant ($\chi^2_1 = 1{\cdot}798$, $P = 0{\cdot}180$). This change indicates larval food availability was more influential for this measure of *V. culicis'* potential horizontal transmission success than being in a co-infection.

Qualitative estimates of potential E. aedis *transmission from larvae and pupae*

The potential for horizontal transmission of *E. aedis* from infected larvae and pupae was estimated as being higher from hosts in the low food treatment (Fig. 1; $\chi^2_1 = 34{\cdot}67$, $P < 0{\cdot}001$). An interaction between parasite treatment and food availability revealed that potential *E. aedis* horizontal transmission from mosquitoes with single infections was higher in low food conditions, and lower in high food conditions, than from mosquitoes in the corresponding co-infection treatment ($\chi^2_1 = 4{\cdot}36$, $P = 0{\cdot}037$). It should be noted that potential horizontal transmission from mosquitoes in the co-infection treatment did not differ across food treatments (Fig. 1).

PARASITE SPORE PRODUCTION IN INFECTED LARVAE AND PUPAE

Longevity of aquatic stage mosquitoes

Larval or pupal survival represents the time available for parasite growth. We found reduced longevity in all infection treatments compared to the uninfected control (Fig. 2a). Among infected larvae/pupae, those with single infections of *V. culicis* survived longer than those with single infections of *E. aedis*, while those infected with both parasites had the shortest survival ($\chi^2_3 = 77{\cdot}149$, $P < 0{\cdot}001$). Figure 2b shows that the cumulative numbers of spores for each parasite were lower in the co-infection treatments.

Vavraia *spore production in larvae and pupae*

The number of *V. culicis* spores was positively related to mosquito survival ($F_{1,37} = 18{\cdot}91$, $P < 0{\cdot}001$) and was reduced by co-infection with *E. aedis* ($F_{1,37} = 5{\cdot}2$, $P = 0{\cdot}029$). Further, an interaction between survival and co-infection reveals, despite lower overall numbers, a higher rate of *V. culicis* spore production in the presence of *E. aedis*, manifested through a steeper slope of spore production with time ($F_{1,37} = 8{\cdot}1$, $P = 0{\cdot}007$) (Fig. 3). There was an issue concerning the interpretation of the interaction between parasite treatment and survival time. This significant interaction term indicates that among models assuming a linear relationship between ln(*V. culicis* spores) and survival time, the best model is the one where the two parasite treatments, single infection and co-infection with *E. aedis*, have different slopes. However, given the distribution of the data points shown in Fig. 3, it could be reasonably argued that there is a nonlinear relationship between ln (*V. culicis* spores) and survival time unaffected by the parasite treatment. To resolve this issue, we used Akaike's information criterion to compare the linear ANCOVA to four nonlinear models. These nonlinear models were a saturating, a nonlinear saturating, a quadratic and a cubic model. The ANCOVA model had the smallest AIC value and thus explains the data better than all the nonlinear models examined (results not shown).

Edhazardia *spore production in larvae and pupae*

The number of *E. aedis* uninucleate spores available for horizontal transmission from larvae and pupae was not influenced by the presence of *V. culicis* ($F_{1,45} = 0{\cdot}1{\cdot}76$, $P = 0{\cdot}191$). The number of uninucleate spores increased with mosquito longevity ($F_{1,45} = 16{\cdot}07$, $P = 0{\cdot}0002$), but

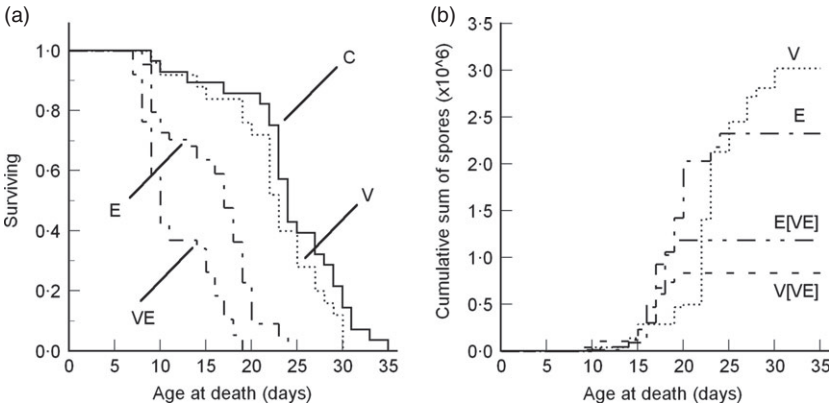

Fig. 2. The (a) longevity of mosquitoes remaining as larvae and pupae in low larval food conditions and (b) the cumulative number of spores produced in each parasite treatment as a function of time.

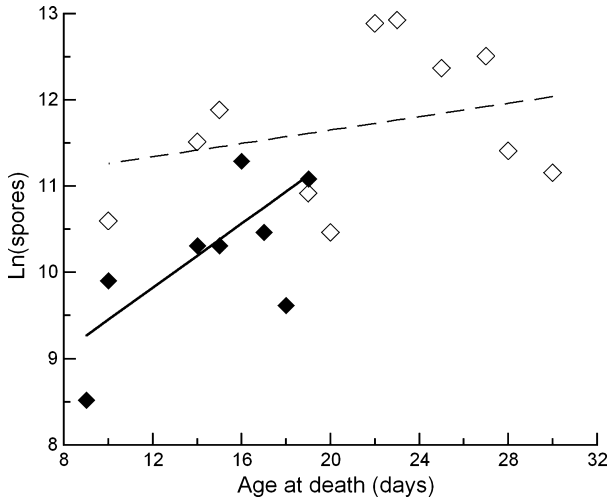

Fig. 3. Number of *V. culicis* spores produced in mosquitoes that died as larvae or pupae in low food conditions as a function of the time during which their host survived. Open circles represent individual mosquitoes with single *V. culicis* infections and closed circles individual mosquitoes with co-infections.

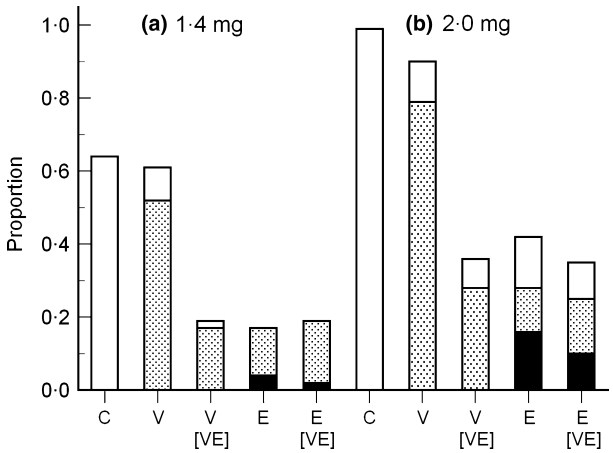

Fig. 4. The proportion of mosquitoes dying as adults, and of those the proportion affording the potential horizontal transmission of *V. culicis* and *E. aedis* (dots) and vertical transmission of *E. aedis* (black shading), at (a) low and (b) high larval food availability in the different parasite treatments. The different parasite treatments are denoted as C = uninfected control, V = *Vavraia culicis* and E = *E. aedis*, and VE indicates the co-infection treatment.

this did not vary between parasite treatments (parasite*longevity, $F_{1,45} = 0{\cdot}107$, $P = 0{\cdot}745$).

Total number of transmissible spores produced

Figure 2b shows the total number of spores produced by infected hosts dying as larvae or pupae in the low food treatments potentially available for horizontal transmission. In the case of both single infections, the number of spores produced by each parasite exceeded the number used to initiate the infections. Single infections of *E. aedis*, seeded with a total of 8000 spores, yielded $2{\cdot}325 \times 10^6$ (95% CI: $1{\cdot}675 \times 10^6$ to $3{\cdot}085 \times 10^6$) spores, that is a multiplication rate of *c.* 300. Single infections of *V. culicis*, seeded with $1{\cdot}6 \times 10^6$ spores, yielded $2{\cdot}965 \times 10^6$ (95% CI: $1{\cdot}940 \times 10^6$ to $4{\cdot}204 \times 10^6$) spores, that is a twofold increase. In the co-infection treatment, both parasites produced a lower yield of spores than in their single infections. For *V. culicis*, the total number of spores produced was approximately half the number used to initiate infections [$8{\cdot}35 \times 10^5$ (95% CI: $5{\cdot}150 \times 10^5$ to $1{\cdot}185 \times 10^6$)], and 3·5 times lower than that produced in single infections. *E. aedis* produced about 150 times more spores than the number used to seed infections in co-infections [$1{\cdot}185 \times 10^6$ ($6{\cdot}75 \times 10^5$ to $1{\cdot}755 \times 10^6$)] such that co-infection halved spore production.

QUALITATIVE ESTIMATES OF PARASITE TRANSMISSION SUCCESS FROM ADULT MOSQUITOES

Qualitative estimates of V. culicis *transmission from adult mosquitoes*

The potential for horizontal transmission of *V. culicis* by infected adults was greater in single infections ($\chi^2_1 = 35{\cdot}16$, $P < 0{\cdot}0001$) and in conditions of high larval food availability ($\chi^2_1 = 8{\cdot}50$, $P = 0{\cdot}0036$). There was no interaction between parasite treatment and food availability when

measuring potential horizontal transmission from infected adults ($\chi^2_1 = 2{\cdot}56$, $P = 0{\cdot}120$) (Fig. 4).

Qualitative estimates of potential E. aedis *transmission from adult mosquitoes*

High larval food availability increased *E. aedis*' potential transmission, both horizontally ($\chi^2_1 = 7{\cdot}94$, $P = 0{\cdot}0048$) and vertically ($\chi^2_1 = 11{\cdot}18$, $P = 0{\cdot}008$). There was no significant effect of *E. aedis* being alone or in co-infection with *V. culicis* for its potential horizontal ($\chi^2_1 = 0{\cdot}54$, $P = 0{\cdot}464$) or vertical ($\chi^2_1 = 0{\cdot}174$, $P = 0{\cdot}676$) transmission. Nor was there an interaction between the food and infection conditions for either type of transmission (horizontal transmission; $\chi^2_1 = 0{\cdot}17$, $P = 0{\cdot}665$, vertical transmission; $\chi^2_1 = 0{\cdot}953$, $P = 0{\cdot}329$) (Fig. 4).

Discussion

In this study, we investigated how co-infection with two different microsporidian parasites and host food availability interact to affect potential parasite transmission via effects on both host and parasite life histories. We found the potential transmission of both parasites was negatively influenced by co-infection, although its effects were quantitatively asymmetric. Host food availability did not modify the effects of co-infection, but was found to independently affect the potential transmission of both parasites by strongly influencing whether mosquitoes died as larvae/pupae or as adults.

CO-INFECTION EFFECTS ON POTENTIAL TRANSMISSION FROM LARVAE AND PUPAE

The impact of co-infection on potential transmission was more complex for *V. culicis* than for *E. aedis*. Indeed, co-infection had no effect on some potential transmission components (probability that hosts will die in the aquatic environment, Fig. 1) yet negatively affected other components [longevity in the aquatic environment (Fig. 2a), spore production (Fig. 2b)] of *E. aedis*. In the case of the potential transmission of *V. culicis*, co-infection had both positive (probability that hosts will die in the aquatic environment, Fig. 1) and negative effects [longevity in the aquatic environment (Fig. 2a), spore production (Fig. 2b)].

The timing of host death is important for quantitative estimates of the potential horizontal transmission as the number of uninucleate spores produced by each parasite accumulated exponentially over time (Fig. 2b). Co-infection negatively influenced the amount of potential horizontal transmission to be gained from hosts dying as larvae or pupae in the low food treatments for both parasites. This is because co-infected larvae and pupae tended to die earlier than those with single infections of either parasite (Fig. 2a), thus allowing relatively less time for the production of horizontally transmitting uninucleate spores. The increased virulence of co-infected vs. singly infected hosts (as measured by larval survival times) reduced the number of spores produced in larvae and pupae infected by *V. culicis* to a greater extent than those infected by *E. aedis* (Fig. 2b).

Our results suggest that co-infection affects *V. culicis*' spore production directly in two ways both through a negative direct effect on the mean number of spores produced (Fig. 2b) and by increasing their relative rate of production (Fig. 3). We do not know what mechanisms act to reduce spore production under co-infection. Possible candidates are competition to infect host cells or to exploit other host resources for metabolic purposes. Resource competition between co-infecting microsporidian species is a likely mechanism, as they extract resources for their metabolic needs exclusively from their host and it is likely they have similar metabolic needs. A recent study found that much of the host proteome response to single infections of these parasites was shared and that co-infection modified this response (Duncan *et al.* 2012).

The second direct effect of co-infection revealed by the steeper slope of spore production with time for *V. culicis* in co-infections is intriguing (Fig. 3). Fellous & Koella (2009) observed a similar result with *V. culicis* producing spores earlier when in co-infections with *Ascogregarina culicis*. Shifts in life history where investment in reproduction occurs earlier have been documented for many hosts infected by parasites when costs of infection increase with time (see e.g. (Michalakis & Hochberg 1994; Agnew *et al.* 1999; Agnew, Koella & Michalakis 2000; Chadwick & Little 2005; Adiba, Huet & Kaltz 2010). Similarly, earlier investment in reproduction (spore production) could be beneficial for *V. culicis* in co-infections with *E. aedis*, as co-infected hosts are likely to die sooner. An earlier investment in spore production might occur at the expense of maximal levels of within-host infection and the number of spores that could ultimately be produced. Nonetheless, it may be an optimal strategy for *V. culicis* to secure some potential horizontal transmission from co-infected hosts rather than none if hosts die before spore production begins.

One way to summarize and evaluate the net effect of co-infections is to investigate their effect on the overall number of spores produced and available for horizontal transmission within breeding sites. As shown in Fig. 2b, the spore output was lower in the co-infection treatment than in the single infection treatments for both parasites. The effect was harsher, however, for *V. culicis,* whose total spore production was divided by *c.* 4 due to the presence of its competitor, than for *E. aedis*, whose total spore production was divided by 2. Further, while *E. aedis* produced more spores than those used to seed the experimental infections in both single and co-infections, *V. culicis* managed to do so only in single infections, its relative yield being negative in co-infections.

CO-INFECTION EFFECTS ON POTENTIAL TRANSMISSION FROM ADULTS

Vavraia culicis can potentially gain horizontal transmission from infected mosquitoes emerging as adults and harbouring uninucleate spores. However, microsporidian spores infecting aquatic hosts are sensitive to desiccation (Becnel & Andreadis 1999). This means any potential transmission is only likely if hosts die in or on a body of water for which we do not have quantitative estimates. Although female mosquitoes must return to water to oviposit, there was no evidence of between-site transmission of *V. culicis* in sites experimentally infected 2 years earlier (Reynolds 1972), indicating that this mode of transmission is not particularly frequent for *V. culicis*. Our results suggest if it is to happen, it is more likely to occur in conditions of good larval food availability, in single infections (Fig. 4). However, neither co-infection nor food availability impacted *V. culicis* spore growth in adults (See Table S4, Supporting information).

Edhazardia aedis can also potentially gain horizontal transmission from infected adults in the same manner as *V. culicis*. However, this is equally unlikely to represent an important source of transmission. Instead, conditions favouring rapid larval development are more likely to result in the vertical transmission of *E. aedis* via the eggs of infected adult females (Agnew & Koella 1999). Although laboratory studies find *E. aedis* reduces the reproductive success of female *A. aegypti*, the efficiency of transmission to the next generation can approach 100% (Becnel, Garcia & Johnson 1995). The presence of *E. aedis* in 17 out of 20 *A. aegypti* breeding sites (Hembree 1979) suggests it is better at exploiting infected adults for transmission among sites than *V. culicis*. Our results suggest co-infection of larvae with *V. culicis* will do little to change this (Fig. 4).

HOST FOOD AVAILABILITY EFFECTS ON POTENTIAL TRANSMISSION

A number of studies highlight that the biotic and abiotic environment can modify the consequences of co-infection. The genotypes of the competing parasite species (Seppala *et al.* 2009; Seppälä *et al.* 2012), parasite dose (Fellous & Koella 2009; Seppälä *et al.* 2012), host age (Cattadori, Boag & Hudson 2008) and food availability (Fellous & Koella 2009, 2010) all shape host or parasite life history in co-infections.

We found that the major effect of host food availability on potential transmission of both parasites acted through effects on host life history and independently of effects of co-infection. Low food availability increases the probability that hosts die in the aquatic environment (Fig. 1). *V. culicis* partially benefited from low host food conditions as lower levels of adult emergence increased opportunities for horizontal transmission from larvae and pupae. The potential type of transmission to be gained by *E. aedis* is strongly determined by whether mosquitoes emerge as adults or not. The potential for horizontal transmission from larvae and pupae was greater in the low larval food treatments (Fig. 1), whereas the high larval food treatments favoured the potential for vertical transmission (Fig. 4). It has previously been shown that *E. aedis* progresses through its life cycle at a constant rate with the type of transmission it is likely to gain being determined by the relative rate at which its host develops (Agnew & Koella 1999). Consequently, vertical transmission is more likely when host larvae develop rapidly and emerge as adults, while slow larval development provides more time for *E. aedis* to complete its life cycle with the production of uninucleate spores (Agnew & Koella 1999). This does not always lead to the highest possible levels of transmission, as this microsporidium greatly reduces female fecundity: it decreases the reproductive capacity of *A. aegypti* populations by *c.* 98% (Becnel, Garcia & Johnson 1995). However, the possibility to be vertically transmitted increases the likelihood of some transmission from infected adults, as well as for persistence through colonization of new sites especially when habitats dry up.

Many parasites, like *E. aedis*, are capable of both horizontal and vertical transmission. Theoretical work has investigated the evolutionary and epidemiological dynamics of competing parasites that use both transmission modes at the population, but not the individual level (Lipsitch, Siller & Nowak 1996). It is likely that within-host competition between parasite species with contrasting modes of transmission is an important evolutionary force. Recent empirical work identified that exclusion of a less virulent parasite capable of both transmission modes, by a more virulent horizontally transmitted parasite, is only avoided when transmission of the former is vertical (Ben-Ami, Rigaud & Ebert 2011). Here, we highlight that *E. aedis* transmission mode is unaffected by co-infection. It would be interesting to see whether the outcomes of co-infection on *E. aedis* fitness were different following vertical transmission, or if in competition with a parasite that limited opportunities for vertical transmission.

Few studies have assessed the transmission potential of parasites from co-infected hosts. To our knowledge, only one study has explicitly shown that co-infections reduce parasite transmission to subsequent hosts (de Roode *et al.* 2005). As we do, two studies report the potential for co-infections to impact parasite transmission using effects on the occurrence and number of transmission stages as a proxy (Fellous & Koella 2009; Ben-Ami, Rigaud & Ebert 2011). Both these studies find that co-infections negatively affect the potential transmission of both players under certain conditions and that the size of effects are asymmetric (Fellous & Koella 2009; Ben-Ami, Rigaud & Ebert 2011). Other studies show co-infection-induced modifications in transmission through changes in host behaviour reducing the probability of predation by a definitive host (Haine, Boucansaud & Rigaud 2005; Dianne *et al.* 2010).

Overall, these studies highlight that co-infection can negatively affect parasite transmission.

Co-infections have attracted much attention for how they affect the evolution of virulence. However, studies are only informative for virulence evolution if they are coupled with measures of parasite transmission (Alizon, de Roode & Michalakis 2013). The few experimental studies linking parasite virulence to transmission concern co-infections with different genotypes of the same species. They show that the most virulent genotype generally wins within-host competition, compatible with the predictions of models where parasites compete for common resources (Bell *et al*. 2006; Ben-Ami, Mouton & Ebert 2008; de Roode *et al*. 2005). Consistent with this, we report that interspecific competition between *V. culicis* and *E. aedis* decreases the potential transmission of both parasites, but the less virulent parasite, *V. culicis*, suffers more through greater reductions in spore production. However, predictions for how co-infection would affect virulence evolution of either parasite requires knowledge about the mechanisms involved in their interaction (Alizon, de Roode & Michalakis 2013). Furthermore, studies across multiple generations with natural transmission where epidemiological feedbacks shape virulence are required to truly understand how co-infection impacts parasite evolution. (Choisy & de Roode 2010; Alizon, de Roode & Michalakis 2013).

Although our results are consistent with the predictions of models predicting competition between parasites for a common, shared resource, we do not know the mechanisms by which *E. aedis* and *V. culicis* interact. Our past research on *V. culicis* has shown that this parasite (i) decreases the amount of resources (sugars, lipids, proteins) in infected hosts (Rivero *et al*. 2007), (ii) elicits the production of antimicrobial peptides (Biron *et al*. 2005) and (iii) might inhibit the NOS pathway (Biron *et al*. 2005). A recent study indicates that part of the host proteome response to single infections of *E. aedis* and *V. culicis* is shared (Duncan *et al*. 2012). Despite this, we do not presently have enough information on the mechanistic processes occurring in single *E. aedis* infections and co-infections. There is an absence of evidence that single *E. aedis* infections reduce adult body size (Becnel, Garcia & Johnson 1995) which would be indicative of a negative effect on host resources. However, this does not preclude the possibility that interactions between co-infecting microsporidia may involve competitive exclusion to infect host cells. The effects of *E. aedis* infection on the host immune system are not established, nor whether the immune system modifications induced by *V. culicis* impact *E. aedis*. Finally, our findings suggest within-host competition between these parasites as *V. culicis* plastically responds to the negative effects of co-infection by an earlier investment into spore production. Although we cannot be sure of the processes involved, those dominating have a net effect giving a relative transmission advantage to the more virulent parasite.

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Data accessibility

Data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.qt14r (Duncan *et al*. 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Experimental design.

Table S1. Number of mosquitoes per treatment surviving longer than day 5 and therefore contributing to statistical analyses.

Table S2. Parasite traits in single and coinfections.

Table S3. Host traits in single and coinfections.

Table S4. The effects of co-infection and larval food availability on *V. culicis* spore growth in adults.