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Co-evolution between mosquitoes and microsporidian transmission strategies

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Le Doyen, Prof. R. Bshary

To my main supporter, still with me

Abstract

Parasite and host impose strong selection on each other. The first causes damages and mortality to the host, while the second responds by reducing the detrimental effects and the intensity and/or success of infection. The resulting co-evolutionary dynamics are profoundly affected by the ecological conditions, for these may influence many aspects of host-parasite interactions including life history evolution, virulence and transmission. It is therefore essential to study and incorporate environmental variation in the field of parasitology to gain an exhaustive understanding of how host and parasite evolve.

In this thesis, a single generation and an evolutionary experimental approach were used to investigate the impact of the ecological and epidemiological conditions on several aspects of host-parasite interactions, with the main focus on parasite transmission strategies. Firstly, it was examined the effect of the availability of resources for the host, timing of infection, and co-infection on the virulence and transmission success of two parasites with conflicting transmission strategy. Next, it was tested how the environment influenced the trade-off between vertical and horizontal transmission in a parasite with a mixed mode of transmission and it was assessed the genetic contribution of the host to its transmission mode. Whether the vertical and horizontal component of this parasite and the associated virulence responded to restriction opportunities, represented by different availability of resources over several generations, was investigated with an evolutionary experiment. Finally, the presence of a plastically parasite-induced response on the recombination rate of the host as a potential cross-generational defence mechanism was explored. The experiments cover many key aspects of host-parasite interactions and emphasize the role of the ecological conditions on shaping these relationships. The results and their implications are discussed in detail throughout the thesis.

Overall, this work highlights the dependence of crucial aspects of host-parasite interactions from the epidemiological and ecological conditions. Disentangling the various forces surrounding these interactions may help us to acquire a better knowledge of how a changing environment may drive the evolution of both host and parasite.

Keywords

Aedes aegypti, horizontal and vertical transmission, virulence, life-history evolution, epidemiology, co-evolution, host-parasite interactions, *Edhazardia aedis*, *Vavraia culicis*.

Résumé

Hôtes et parasites s'imposent mutuellement une très forte sélection. Les premiers causent dommages et mortalité aux seconds, qui y répondent en essayant de réduire les effets négatifs, l'intensité et/ou le succès de l'infection. Les dynamiques co-évolutives qui en résultent sont profondément affectées par les conditions écologiques, qui peuvent influencer plusieurs aspects des interactions entre hôtes et parasites, comme l'évolution des traits de vie, la virulence et la transmission. En conséquence, l'incorporation et l'étude des variations de l'environnement dans le domaine de la parasitologie reste essentielle si l'on veut atteindre une compréhension exhaustive de l'évolution de l'hôte et de son parasite. Dans cette thèse, une approche expérimentale a été utilisée pour examiner l'impact des conditions écologiques et épidémiologiques sur plusieurs aspects des interactions entre un hôte et son parasite, avec une attention particulière sur les stratégies de transmission du parasite. En premier lieu, nous avons examiné l'effet de la variabilité des ressources dans l'hôte, du moment/ la chronologie de l'infection, et de la co-infection sur la virulence et le succès dans la transmission de deux parasites dotés de stratégies de transmission évolutivement en conflit. Ensuite, nous avons testé de quelle manière l'environnement et la génétique de l'hôte influencent le trade-off entre transmission verticale et horizontale dans un parasite caractérisé par la capacité d'accomplir les deux types de transmission. En élaborant une expérience évolutive, nous avons évalué si la composante verticale et horizontale de ce parasite et la virulence qui leur est associée répondent aux contraintes écologiques représentées par une disponibilité variable de ressources sur plusieurs générations. Dans une dernière expérience, nous avons investigué la présence d'une réponse plastique du taux de recombinaison de l'hôte induit par le parasite; un mécanisme qui pourrait représenter une défense intergénérationnelle de l'hôte contre une infection parasitaire. Les expériences présentes dans cette thèse couvrent plusieurs aspects des interactions entre hôtes et parasites et mettent l'accent sur le rôle des conditions écologiques dans l'évolution de ces relations. Les résultats et leurs implications sont discutés en détail tout au long de la thèse. Globalement, ce travail de thèse met en évidence la dépendance des interactions entre hôte et parasite des conditions écologiques et épidémiologiques. Réussir à séparer les différentes forces qui entourent ces interactions peut nous aider à mieux comprendre comment un environnement en constante mutation influence et détermine l'évolution des deux contendants, l'hôte et le parasite.

Mots-clés

Aedes aegypti, transmission horizontale et verticale, virulence, évolution de l'histoire de vie, épidémiologie, co-évolution, interactions hôte-parasite*, Edhazardia aedis*, *Vavraia culicis*.

Contents

Chapter 1

General Introduction

1.1 Background

Parasitism is a powerful selective force that acts in virtually all organisms. Parasites, broadly defined as infectious agents causing diseases (from helminths to fungi, bacteria and viruses), are ubiquitous in nature with no organism too small to be a host, not even viruses (La Scola et al. 2008). For example, the fish species *Gasterosteus aculeatus* and *Pungitius pungitius* were found to be parasitized by respectively 51 and 16 different parasites (Morozinska-Gogol 2006), and 1400 species has been described to infect humans (Woolhouse & Gowtage-Sequeria 2015). One of the first cause of mortality in human populations worldwide is due to a parasitic infection (Vitoria et al. 2009), with more than 400,000 deaths caused by malaria in 2015 (WHO 2015).

The interactions between host and parasite affect nearly all biological aspects, including genetic diversity (Liersch & Schmid-Hempel 1998; Whitehorn et al. 2010), the evolution of life history traits (Hochberg et al. 1992), the maintenance of sexual reproduction (Lively 2010), social behaviour (Ezenwa 2004; Ezenwa et al. 2016), and community composition (Pedersen & Fenton 2007). Host and parasite evolve in response to each other's pressure in a constant scenario of co-evolutionary relationships (Hall et al. 2011). Parasites trying to optimize their transmission success will cause severe damages to the host, decreasing their competitive abilities. Parasites may also modify the direction of the genetic correlations among host's traits and determine the response of the host to selection (Ebert & Herre 1996; Koella et al. 1998). On the other hand, the host will respond to reduce the detrimental effects of the infection by enhancing the immune response and the relative mechanisms. Alternatively, the host may change the behaviour or plastically modify its life history traits (Pigliucci 2001), from the anticipation of the age at maturity (Restif et al. 2001) to the increase of their recombination rate (Singh et al. 2015). Offspring with novel genetic combinations are expected to be partially protected from being infected by the same parasites which were able to avoid the genetic resistance of the parents (Jokela et al. 2009; Vergara et al. 2014). The results of these interactions establish the outcome of selection and affect the direction of evolution (Koella & Restif 2001).

Hence, parasites impose strong selection on their host by causing mortality, severe damages and major costs in fitness. Why some parasites harm or kill their hosts, even if they depend on them for their survival and transmission, while others are relatively benign is a long-debated topic in evolutionary biology. Virulence determine a parasite's damage to a population of hosts, and it is one of the central ideas about the evolution of parasites (Gandon et al. 2001; Leggett et al. 2013). A trade-off between virulence and rate of transmission is usually assumed, and virulence is explained as an unavoidable side-product of selection for higher parasite density in the host and consequently more transmission (Anderson & May 1982; Alizon et al. 2009). In line with this assumption, an increase of parasite transmission can be achieved with a higher reproductive rate, but only at the cost of harming the host

more and therefore decreasing both host and parasite longevity. The balance between the costs and the benefits should lead the parasites evolving towards an optimal level of virulence being the one which maximizes their total transmission. The general theory about virulence evolution has been intensively studied (Frank 1996; Bull & Lauring 2014), and it provides a common framework in which comparing experimental and theoretical results. A positive relationship between virulence and transmission has been found in several systems (Mackinnon & Read 2003; Jensen et al. 2006; de Roode et al. 2008; Doumayrou et al. 2013), and a few of them further showed the existence of an optimal level of virulence (Jensen et al. 2006; de Roode et al. 2008). For virulence and transmission are strictly associated, virulence is expected to drastically differ according to the mode of transmission (Antonovics 2017). Indeed, two modes of transmission with distinct levels of virulence can be distinguished, horizontal and vertical. The first one is the spread of the parasite among hosts in a population. It is expected to be related to high virulence when the survival rate of the host is low, or the exploitation of the host increases parasite's transmission rate. The second one is the transmission from mother to offspring and is usually associated to low virulence (high survival rate and fecundity of the host), as host and parasite share their evolutionary interests (Frank 1996).

The observed variability in virulence and transmission is determined by the interaction between host and parasite genotypes (Saulvaudon et al. 2005; Lambrechts et al. 2006), but this needs to be integrated in an ecological framework, accounting for variation in the environmental conditions (Thompson 2006; Gandon & Nuismer 2009; Marchetto & Power 2017). In fact, despite having genetic basis, host-parasite interactions likely depend on ecological and epidemiological factors such as the presence of inter or intra-specific competition (coinfection) (Alizon et al. 2013), the timing of infection (Hoverman et al. 2013), and host's resources (Fellous & Koella 2010; Duncan et al. 2015). It is known that under changing ecological circumstances host's life history traits like growth, survival and reproduction differ significantly (Stearns 1992), and these may alter the selection on the parasites. Environmental heterogeneity has therefore a critical role on the outcome of host-parasite associations. For example, infections by several strains or species of parasites are common (Malpica et al. 2006; Lagrue et al. 2007; Balmer & Tanner 2011) but the virulence expressed by the parasites in single infections rarely predict the outcome of infection by their combination (Louhi et al. 2015). The within-host competition may result in a strong asymmetry and affect the evolution of both parasite and host (Syller 2011; Natsopoulou et al. 2015). In addition, the first parasite to infect the host might exclude the subsequent parasite or let it face a pre-stimulated immune response (Alizon 2012).

The availability of resources of the host is another important aspect influencing the dynamics of host-parasite association and infectious diseases (Lazzaro & Little 2009; Wolinska & King 2009; Shetty 2010), for this may affect the within-host growth of the parasite (i.e. transmission and virulence) and host's defence. In presence of high levels of resources, we may expect a more effective immune response

and ability of the host to clear or resist parasite infection (Koella & Sorense 2002). Hosts developing in detrimental conditions, due to scarcity of food, may be more vulnerable to parasites and experience a higher cost of parasitism (Moret 2000; Ferguson & Read 2002). In contrast, from parasite's perspective, the well-nourished host may represent a better environment as the resources from the host may be used to sustain its own development (Bedhomme et al. 2004; Brown et al. 2010). This was found in the bacterial parasite *Pasteuria ramosa* which increased the transmission rate at increased level of food availability of its host *Daphnia magna* (Ebert et al. 2010; Vale et al. 2013). Thus, virulence and transmission, two of the key traits of parasite's fitness, are highly affected by the ecological settings.

The evolutionary and epidemiological concepts illustrated above, have been developed primarily for parasites with exclusive vertical or horizontal transmission. However, the two forms of transmission are not mutually exclusive, and they can coexist under certain circumstances (Lipsitch et al. 1996; Ferdyn & Godelle 2005). Many parasites indeed present a mixed-mode of transmission and are able to do both. Several parasites usually classified as horizontally transmitted further have a vertical component of transmission during their life cycle, and they may be additionally transmitted between mother and offspring (i.e. transmammary, transovum, transovarial, transplacental, intrauterine transmission).This is the case of the medically important viruses HIV, human papilloma virus, and hepatitis B and C viruses, but several bacteria, protozoa and microsporidia similarly present both mode of transmission (Ebert 2013). Horizontal and vertical transmission can be positively correlated but it is rather unusual (Kover et al. 1998; van Frankenhuyzen et al. 2007; de Roode et al. 2009). It is common that in these parasites the two modes of transmission are physiologically, developmentally or evolutionarily linked (Ebert & Mangin 1997; Messenger et al. 1999; Magalon et al. 2010; Dusi et al. 2015), and the investment in one transmission mode (either vertical or horizontal), will reduce the transmission with the other. The trade-off between horizontal and vertical transmission is central to these parasites and highly affect their evolution (van Baalen 2000). The two modes of transmission and the associated virulence might be favoured under different circumstances and the selective pressure experienced might be context-dependent. Some experimental evolution studies manipulated the opportunities for the two transmission strategies of a mixed-mode transmission parasite over many generations. Rapidly growing populations of the host selected for increased vertical transmission of a bacterial parasite (Magalon et al. 2010) which almost loss its horizontal virulent component (Dusi et al. 2015), suggesting that specific environmental conditions may drive parasites to change their virulence by switching their transmission strategy.

Other empirical works on parasite with mixed mode of transmission show how host's conditions affect parasite's transmission strategy and the trade-off between vertical and horizontal transmission. One example is the bacterium *Holospora undulata* that infects the ciliate *Paramecium caudatum*. The bacterium can be transmitted horizontally through an infectious stage, but it also has a reproductive

stage where infects the daughter nuclei of infected cells (vertical transmission). In a situation enabling high replication rates of the host the reproductive stage is favoured, and the parasite is passed on vertically to the daughter cells. In contrast, the parasite invests more into the infectious forms and is transmitted horizontally at low population growth rate (Kaltz & Koella 2003). Another example is the microsporidian parasite *Edhazardia aedis* that infects the mosquito *Aedes aegypti*. The parasite has two specific forms of spores specialized in either vertical (binucleate spores) or horizontal (uninucleate spores) transmission. Vertical transmission occurs when infected females lay the eggs, horizontal when infected larvae die in the water releasing spores which will infect other larvae. When host's conditions deteriorate and the emergence into adults is delayed, horizontal transmission becomes more likely (Koella et al. 1998; Agnew & Koella 1999). The studies suggest that only good food conditions enable the host to emerge as adult and lay eggs, and therefore the parasite to transmit vertically. Low food conditions slow larval growth, making them more attractive for horizontal transmission (Koella et al. 1998; Agnew & Koella 1999).

Transmission is a fundamental components of parasite fitness, and it considerably influences the epidemiology and the evolution of host-parasite interactions. Since mode of transmission and virulence are intrinsically linked, ignoring how transmission mode evolves and the ecological conditions (such as resources, competition or host's genetics) affecting its evolution, highly limits our understanding of the co-evolutionary dynamics between hosts and parasites. A better comprehension of the variables determining the relative importance of vertical and horizontal transmission may help to predict the direction of parasite evolution, the associated virulence, and finally the management of parasites. The potential for an evolutionary conflict over the modes of transmission in parasites capable of vertical as well as horizontal transmission offer an excellent opportunity to study the ecological factors selecting for one or the other strategy. Considering the natural variation in space and time of host and parasite's environment, it remains essential to investigate and incorporate such variation in the field of evolutionary parasitology.

1.2 Thesis introduction

Here, I studied the impact of the ecological and epidemiological conditions on host-parasite interaction, principally focusing on parasite transmission strategies. I used the mosquito species *Aedes aegypti* as host, and two microsporidia parasites. *Edhazardia aedis*, a parasite with a mixed mode of transmission, was used to empirically test how the environment influence the evolution of vertical and horizontal transmission, and how the natural virulence of the two transmission components affected host's life-history traits. I used *Vavraia culicis* to examine within-host competition and the plastic genetic response of the host to infection. These results may have general biological implications considering the potential for ecological and evolutionary feedbacks. They may also expand our

knowledge of host-parasite interactions, helping to develop new models and support the theoretical works. Further, since *Ae. aegypti* is an important vector of many human parasites, the results may be relevant for public health and in the management of parasites, for microsporidia has been proposed as biological control of mosquitoes (Koella et al. 2009; Lorenz & Koella 2011).

1.2.1 Parasite's transmission strategy in multiple infections

Multiple infections by several strains or species of parasites are the rule rather than the exception in nature (Read & Taylor 2001; Balmer & Tanner 2011), and they have been reported in bacteria, plants, and animals including humans (Lord et al. 1999; López-Villavicencio et al. 2007; Rutrecht & Brown 2008; Turner & Duffy 2008). They can hinder the development of vaccines and lead to selection for antibiotic-resistant strains (Mackinnon et al. 2008; Read & Mackinnon 2008; Balmer & Tanner 2011). From an evolutionary perspective, multiple infections may modify the selective pressure and influence the outcome of the infection with changes in transmission and virulence (Mideo 2009; Alizon et al. 2012; Alizon 2013). Indeed, conflicting life-history strategies such as transmission mode or within-host growth may affect the evolution of both parasite and host (Syller 2011; Natsopoulou et al. 2015). Parasite with contrasting modes of transmission (e.g. horizontal vs mixed-mode transmission) have different evolutionary interests and they impose strong selection on each other. The availability of resources of the host (Fellous & Koella 2010) and the order of infection (Hoverman et al. 2013; Marchetto & Power 2017) are likely to have an important role in determining the result of multiple infections. High level of host's resources can either cause less or more competition between the parasites, depending on whether the competition is direct or indirect via the host's immune response (Balmer et al. 2009; Alizon et al. 2012). Being the first parasite to infect, has the time advantage to fully develop and exploit the host (Hood 2003; Paul et al. 2004). Further, the virulences in single infections is rarely predicting the results of their combination (Louhi et al. 2015), and selection will favour the most competitive strains or species. Multiple infections are therefore a major concern in evolutionary ecology and human health. In the second chapter, I aim to study the transmission strategy of two competing species of parasites (*Vavraia culicis* and *Edhazardia aedis*) that share the same route of infection but have different mode of transmission. I additionally analyse how resource availability and time of infection affect host's life history traits in the context of multiple infections. Since these interactions are rather common, it is important to investigate and study the factors that may lead to unexpected epidemiological outcomes.

1.2.2 Trade-off between vertical and horizontal transmission

Transmission mode is a major component of parasite fitness which is strictly related to other life-history traits like growth rate, developmental time, reproduction and virulence (Alizon 2009). Horizontal transmission in usually associated with high levels of virulence (exploitation of the host to increase the transmission stage and the rate of transmission), while vertical transmission being linked to host's fecundity results less virulent (Lipsitch et al. 1996). In parasites with mixed-mode of transmission the investment into either vertical or horizontal transmission implies a reduction in the alternative strategy (Ebert 2013; Antonvics 2017). Although several studies found a trade-off between the two forms of transmission and how this was affected by the environmental settings, little is known about its genetic basis. The specific match of host's and parasite's genotype mediates life-history traits and resistance against infections (Casadevall & Pirofski 2003; Lambrechts et al. 2006) and are likely to govern the transmission trade-off in parasites with a mixed mode of transmission. A different genetic background of a parasite's host may control parasite traits (Agnew & Koella 1999; Tintjer et al. 2008) including transmission mode and therefore push the parasite towards different evolutionary trajectories. Parasite infecting hosts with different genetic background will be subjected to specific selective pressure. In this chapter, I investigated whether high and low food availability and the genotype of the mosquito *Aedes aegypti* (represented by full-sib families) affected the transmission mode of the parasite *Edhazardia aedis*. The study contributes with empirical evidence to a better understanding of the mechanisms and the trade-offs leading to different evolutionary pathways in transmission modes. Moreover, this work considers how the genetic variation in the host rather than in the parasite can considerably influence the epidemiology or drive the evolution of the transmission mode in the parasite. Due to the natural difference in virulence of the horizontal and vertical transmission, host's contribution to transmission mode is rather important as it may determine the levels of virulence.

1.2.3 Evolutionary changes in vertical and horizontal transmission

The ecological and epidemiological conditions greatly affect the evolution of the transmission mode and consequently of parasite virulence. The changing environment, including resource availability, is a main effector of evolutionary transition, for example from parasitism to mutualistic association (Dusi et al. 2015). High level of nutrients may enhance the development of the host, while malnutrition slow host's growth. However, the influence of these factors on one or the other mode of transmission and their consequences for parasite's evolution received little attention. Mixed-mode transmission parasites represent a rare opportunity to investigate the relative importance of vertical and horizontal transmission under different ecological conditions, for these may promote evolutionary divergence of parasite life-history traits (Magalon et al. 2010; Ebert 2013). In the fourth chapter, I aim to study the role of host's resource availability in the evolution of parasite transmission, and how this is reflected

on the life-history traits of the host *Aedes aegypti* which was not allowed to evolve. After 10 generations of evolution, I tested whether the horizontal and vertical transmission components of *Edhazardia aedis* responded to environmental restriction opportunities. Long-lasting experiment are important to directly test and observe the theoretical predictions. This approach allowed us to measure the impact of different ecological settings on the evolution of parasite's transmission and virulence.

1.2.4 Genetic recombination as a defence trait

Sexual reproduction through the random assortment of the chromosomes and meiotic recombination may tear apart favourable genetic combinations (Agrawal 2006; Hartfield & Keightley 2012). Despite this potential cost, shuffling genes might help populations to a faster adaptation to a changing and novel environment represented for example by parasites. This idea is often used in a co-evolutionary scenario of fluctuations of allele frequencies between host and parasite (Decaestecker 2007; Gandon et al. 2008; Gaba & Ebert 2009), and it underlies the link between parasitism and the maintenance of sexual reproduction and recombination (Lively 2010). Alternatively, the infected parental generation, may be able via a plastic response to actively modify the recombination rate in order to diversify their progeny. The offspring with sufficiently unfamiliar genotypes will be partially protected from being infected by the same parasite that overcome the genetic defences of the parents. Since infected hosts are favoured by producing offspring with rare and unusual genetic combinations, selection will tend to maintain a high recombination rate in populations where parasites are common (Jokela et al. 2009; Vergara et al. 2014). The plasticity in recombination may be an overlooked mechanism for the host to protect the offspring against its parasites without directly involving the immunity pathways. Nevertheless, the 2 studies to date in animal systems gave conflicting results (Singh et al. 2015; Dumont et al. 2015). In this fifth chapter, I tested whether the females of *Aedes aegyti* increased their recombination rate after a parasite infection with the microsporidia *Vavraia culicis*. The results have important consequences for the genetic diversity and the adaptability of host and parasite populations, and how these may co-evolve. The data may have relevant implications for the ecological and evolutionary feedbacks between the vector *Aedes aegyti* and its arboviruses infecting human populations (i.e. Zika, Dengue, Chikungunya).

1.3 Experimental system

I used the mosquito species *Aedes aegypti* as host and two microsporidia parasites with different life cycle, *Edhazardia aedis* and *Vavraia culicis*.

1.3.1 The mosquito *Aedes aegypti*

Aedes aegypti, the main vector of yellow fever (Tomori 2004) and arboviruses (Petersen et al. 2016), is a ubiquitous mosquito in tropical and subtropical regions. Its life cycle goes through a holometabolous development composed by four distinct stages, egg, larva, pupae and adult (Figure 1). The adult is the only non-aquatic stage, and the females require a blood meal to lay the eggs. Its genetics, physiology, and ecology have been extensively studied and are well known due to its impact on human health (Christophers 1960; Nene et al. 2007). The larvae of *Aedes aegypti* may experience a wide range of growth conditions and environmental fluctuations including interspecific competition (Reiskind & Lounibos 2009) in either natural or artificial containers (Chan et al. 1971), leading to a developmental period ranging from 6 to 30 days (Southwood et al. 1972). Two aspects that make it suitable for lab work is that its eggs can be stored for a long period and that a single mating results in enough sperm to let the female lay eggs throughout her life (Munstermann 1997, Oliva et al.2014). Further, the hatching of the eggs can be synchronized using conditions of partial vacuum (Christophers 1960), which permits to have experimental individuals of the same age. For all the experiments reported in this thesis we used the UGAL strain of *Ae. aegypti* provided by Patrick Guérin (University of Neuchâtel), an undocumented strain that was established in the 1970s (Kuno 2010).

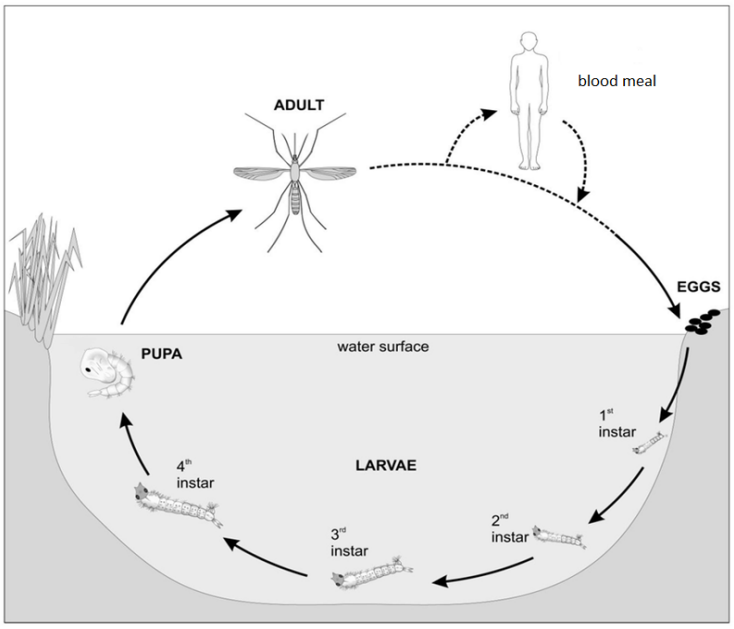

Figure 1 Life cycle of the mosquito. During its development the mosquito goes through four separate and distinct stages: egg, larva, pupa, and adult. The larva and pupa represent the juvenile stage which occurs in the water, the adult is the only non-aquatic stage. The mosquito species *Aedes aegypti* require a blood meal to lay the eggs.

1.3.2 *Edhazardia aedis*

Edhazardia aedis is a specific parasite of *Ae. aegypti* with a complex life cycle (Figure 2A) involving horizontal and vertical transmission (Becnel et al. 1989; Becnel et al. 1995; Johnson et al. 1997; Desjardins et al. 2015). Two types of spores can be morphologically and functionally distinguished: uninucleate spores for horizontal transmission and binucleate spores for vertical transmission. The larvae of the mosquito are infected when they ingest uninucleate spores suspended in water. After a first period of development, the parasite produces binucleate spores in adult females which are responsible for vertical transmission (transovarial transmission). Males provide no opportunity for vertical transmission, so adult males are a dead-end for the parasite. Once the infected eggs hatch, the parasite develops into uninucleate spores. These vertically infected mosquitoes die as juveniles and release the uninucleate spores into the aquatic environment where they can remain infective for about 48 hours (Nasci et al. 1992). The uninucleate spores are eaten by larvae to complete the life-cycle. Thus, larvae that acquire the spores horizontally usually emerge as adults and transmit vertically, while larvae that are infected vertically do not survive beyond pupation. Occasionally, as illustrated in Figure 2A, the parasite life cycle deviates from this description so that the usual alternation of vertical and horizontal transmission is broken. The horizontally infected larvae die before emergence, and this may result in a second round of horizontal transmission without the requirement of vertical transmission (Becnel et al 1989). The parasite has therefore switched from uninucleate to binucleate and then back to uninucleate spores. Thus, underlying this sequence of transmission, the parasite has a fixed developmental sequence with the alternation of binucleate and uninucleate spores (Becnel et al 1989; Johnson et al. 1997), but not necessarily with a fixed timing.

1.3.3 *Vavraia culicis*

Vavraia culicis is an obligate endocellular parasite, which can persist in the aquatic environment outside the host cell and infects larvae when they ingest the spores with their food. The ingestion of the spores results in the infection of the gut and epithelial cells, from where the infection spreads to the fat body and other tissues (Andreadis 2007; Desjardins et al. 2015). After the infection, parasite transmission mainly occurs in two ways (Figure 2B). Firstly, it can be transmitted horizontally among larvae when the infected larvae die, releasing the spores into the water (Agnew et al. 1999; Becnel et al. 2005). A condition-dependent expression of virulence due to low availability of food or strong infection and a positive relation between virulence and transmissibility have been found in a previous study (Bedhomme et al. 2004). Secondly, the larvae survive long enough and emerge into adults, these will eventually die and release the spores in the water which will infect new larvae. In addition, it can be transmitted by adhering to the eggs of an infected female (transovum transmission) and thus reaching a new larval site (Andreadis 2007).

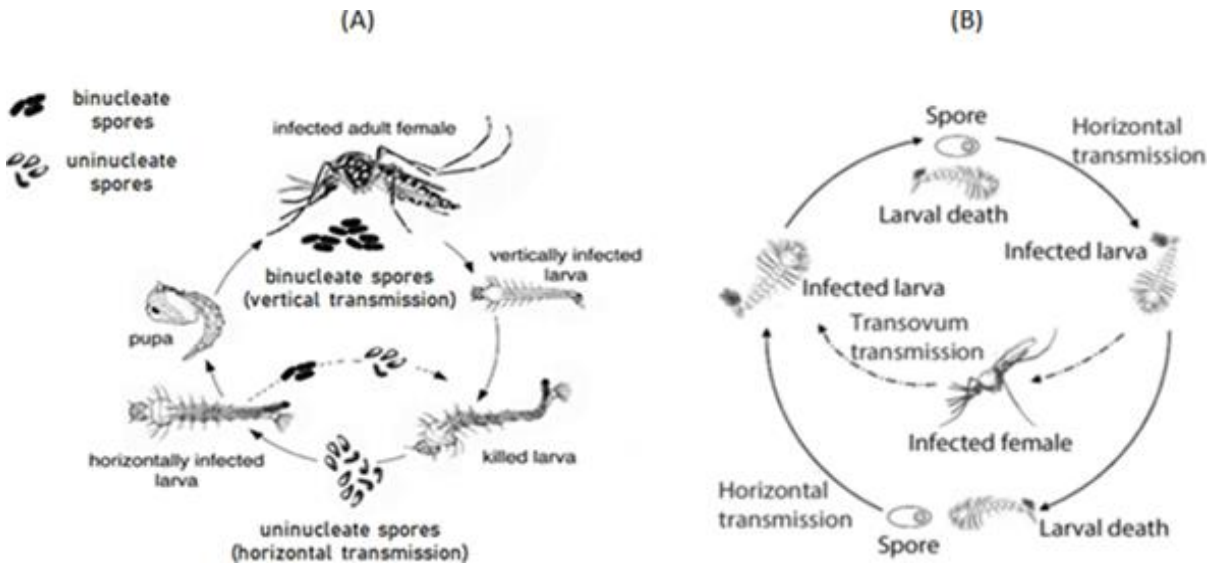

Figure 2 Life cycle of *Edhazardia aedis* (A) and *Vavrais culicis* (B). *Edhardia aedis* is a mixed- mode transmitted parasite with a fixed developmental sequence. It alternates the production of binucleate spores transmitted with the eggs laid by infected females (vertical transmission), and uninucleate spores transmitted in the water among larvae at juvenile's death (horizontal transmission). *Vavraia culicis* has a strictly horizontal transmission that occurs among larvae or from adult to larvae when an individual, adult or juvenile, dies in the water releasing the spores. Deviations from these life cycles are represented with dashed arrows and are described in the main text.

1.4 Research aims

The overall goal of this PhD is to investigate the effect of epidemiological and environmental factors on the co-evolutionary dynamics between the mosquito *Aedes aegypti* and two microsporidian parasites with different mode of transmission; the horizontally transmitted *Vavraia culicis* and the mixed-mode transmitted *Edhazardia aedis*. In this thesis I used a single generation and an evolutionary experimental approach to gain a better understanding of host-parasite interactions.

The thesis is based on four main projects reported in the following chapters:

- **Chapter 2**

  The outcome of co-infection of two parasites with conflicting transmission strategy; the role of the mode of transmission, host's resources, and time at infection in single and multiple infections.

- **Chapter 3**

  The effect of resources on parasite transmission strategy and description of a genetic basis in the host influencing the trade-off between vertical and horizontal transmission.

- **Chapter 4**

  Analysis of the evolution of the vertical and horizontal transmission component of a parasite with a mixed mode of transmission under different host's resource availability.

- **Chapter 5**

  Investigation of a plastically parasite-induced response on the recombination rate of the mosquito *Aedes aegypti*.

Chapter 2

Timing of co-infection and transmission mode drive parasite competition in multiple infections

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2.1 Abstract

How co-infection affects the evolutionary pressures on a parasite's virulence depends on how each parasite affects its competitor's transmission, how the co-infection affects the host's mortality and how these two effects differ among epidemiological and ecological conditions. We investigated the effect of co-infection, timing of infection and availability of food for the host on the transmission success and virulence of two microsporidian, Vavraia culicis and Edhazardia aedis, that infect the mosquito Aedes aegypti but differ in transmission routes: V. culicis is mainly transmitted horizontally among larvae while E. aedis can be transmitted among larvae or vertically from females to their eggs. Low availability of food increased juvenile mortality, delayed age at emergence and enabled horizontal transmission among larvae, particularly with early infections. Although this was true for both parasites, the virulence and the probability of horizontal transmission additionally increased for V. culicis in multiple infections. High resources and late infection reduced juvenile mortality and favoured the probability of emergence into adults. For both parasites, the transmission potential from adult to larvae increased with early infection, but it further depended on the presence of the competitor for V. culicis. The transmission potential of V. culicis was highly reduced when Ed. aedis was already established in the host (early infection). Further, the longevity of the adults during co-infection decreased (higher virulence) for V. culicis, but this was not followed by a higher probability of transmission despite its transmission require host's death. For Ed. aedis, adult's mortality increased with early infection but not with co-infection. In addition, its transmission potential was similar in single or multiple infection, suggesting that its impact on the adults was not influenced by V. culicis. Our results show how transmission and virulence were highly affected by co-infection, order of infection, and host's resources, and that the interactions between these factors may lead to complex epidemiological outcomes.

2.2 Introduction

Being infected by several strains or species of parasites is common (Petney & Andrews 1998; Read & Taylor 2001; Balmer & Tanner 2011). Multiple infections have been reported in bacteria (Turner & Duffy 2008), plants (Meijer & Leuchtmann 1999; Malpica et al. 2006; López-Villavicencio et al. 2007) and animals (Lagrue et al. 2007; Rutrecht & Brown 2008; Pinset et al. 2017) including humans (Griffith et al. 2011). It has been observed, for example, that most of the people infected by malaria harbour more than five clones of *Plasmodium falciparum* (Lord et al. 1999), that several dengue serotypes are found in nearly 20 % of the infections (Bharaj et al. 2008), and that more than 73 % of infected people carry more than one helminth species (Fleming et al. 2006). As multiple infections can modify the selective pressure on the parasites and influence the outcome of the infection (Alizon et al. 2012; Alizon 2013), they are a major concern in evolutionary ecology and for human health.

Multiple infections can, for example, select for parasites with greater or less virulence (i.e. parasite-induced mortality rate of the host) (Alizon et al. 2012; Sofonea et al. 2017). The underlying idea is based on the trade-off between components of the parasite's fitness (Anderson & May 1982; Frank 1996; Alizon et al. 2009), principally between virulence and rate of transmission. A parasite can increase its transmission rate, for example by increasing its rate of replication and its load, but only at the cost of increasing the host's mortality rate, thereby decreasing the time available for transmission (Mackinnon & Read 2003; Salvaudon et al. 2005; de Roode et al. 2008; Doumayrou et al. 2013). Critical features of these ideas are (i) how the virulences of the individual strains combine to determine the mortality rate of the multiply infected host (Bremermann & Pickering 1983) and (ii) how the transmission rate of a parasite is affected by its competitor.

The virulences in single infections rarely predict the outcome of infection by their combination (Louhi et al. 2015), for the mortality rate of a multiply infected host is determined by details of the interaction between the parasite genotypes and the host (Schjørring & Koella 2003; Mideo 2009; Seppälä et al. 2012). Thus, the overall mortality rate can be higher than that of the most virulent parasite (de Roode et al. 2005a), lower than that of the least virulent parasite (Inglis et al. 2009), or in between the two (Harrison et al. 2006). Since research on the virulence caused by multiple infections is crucial, a better understanding of the parasites' strategies to win within-host competition and the resulting impact on their relative transmission success is needed (Antonovics et al. 2017). This is especially important when considering different parasite species with conflicting life-history strategies such as the transmission mode. These interactions may result in a strong asymmetry in competitive success and affect the evolution of both parasite and host (Syller 2011; Natsopoulou et al. 2015).

Parasites with contrasting modes of transmission infecting the same host have different evolutionary interests and they impose strong selection on each other. Horizontally transmitted parasites (transmission between unrelated hosts) are usually virulent, if within-host growth, and thus host exploitation, increases the transmission rate. In contrast, vertically transmitted parasites (transmission from infected parent to offspring) are expected to be less virulent, for their transmission is linked to their host's reproductive success (Fine 1975; Frank 1996). Confusing these ideas, some parasite species may use a mix of horizontal and vertical transmission. Although multiple infections often occur with parasites that have mixed-mode transmission and contrasting transmission strategies (Ebert 2013), little empirical work has explored the epidemiological and evolutionary consequences. The previous studies highlighted the effect of multiple infection between parasites with strictly horizontal and vertical transmission (Haine et al. 2005; Jones et al. 2010), or between parasites with strictly horizontal and mixed-mode transmission (Ben-Ami et al. 2011).

The outcome of coinfection is likely to depend on further factors, such as timing of infection (Hoverman et al. 2013; Marchetto & Power 2017) and host's resources (Agnew & Koella 1999; Fellous & Koella 2010; Duncan et al. 2015). The parasite infecting first is usually favoured as it has more time to develop before being influenced by the competitor (Hood 2003; Paul et al. 2004; de Roode et al. 2005b). Its later competitor, in contrast, is faced by an infection that has already used the host's resources and pre-stimulated immune responses. The first parasite can therefore prevent further infections through competitive exclusion (Izhar et al. 2015) or it can drastically reduce the effect of a second invasion (Kim et al. 2010).

Since host's resources can modify host-parasite interaction, we may expect that greater availability of resources leads to less competition and, consequently, less interference between two parasites (Alizon et al. 2012). However, if most of the competition is indirect, via the immune response, we might expect that a greater availability of resources, which enable a more effective immune response, leads to stronger competition (Balmer et al. 2009).

Here, using the mosquito *Aedes aegypti* and the two microsporidians *Vavraia culicis* and *Edhazardia aedis*, we examined how the presence or absence of a parasite's competitor, the timing and the sequence of infection and the host's food conditions influence the outcome of infections. Our main goal was to study how these variables affected the transmission strategy of each parasite and several life-history traits of the host. *V. culicis* is a strictly horizontally transmitted parasite, whereas *E. aedis* presents a mixed-mode transmission and can be transmitted horizontally or vertically following a developmentally fixed program. Horizontal transmission for both parasites occurs when juvenile individuals die in the aquatic environment and the uninucleate spores released in the water spread among other juveniles. For *V. culicis* transmission is also possible from adults to larvae, for the spores

can stick to the eggs and are then transferred to the breeding site when the eggs are laid. Vertical transmission of *E. aedis* involves the production of binucleate spores infecting a female's eggs.

We expected that food restriction would slow host's development and delay emergence, enhancing horizontal transmission among larvae in both parasites (Agnew & Koella 1999, Bedhomme et al. 2004). Since the two microsporidia compete within the same tissue at the start of the infection (Andreadis 2007), we expected that the parasite infecting first would have an advantage over the competitor. Further, as we know of no effective immune responses of mosquitoes against microsporidia, we expected that competition would be for resources, so that food restriction would enhance the effect of co-infection. We therefore expected that a competing parasite would induce a parasite to invest more in transmission among larvae, so that we would observe greater virulence in multiple infections. In high levels of food and with late infections we expected a higher proportion of individuals emerging and a faster development into adults. Similarly to the among larvae transmission, we expected an early infection to favour the within-host growth of the parasite and the transmission from adult to larvae. In addition, we expected higher virulence and a reduced longevity of the adults in the case of co-infection. Finally, we expected that the contrasting transmission mode of *E. aedis* and *V. culicis* from adults to larvae, respectively vertical and horizontal, would reduce and affect the transmission potential of the competitor. In particular, as *E. aedis* is a specific parasite of *Ae. aegypti*, we expect it to have a greater impact on the mosquito by driving the within-host competition in the adults and imposing its transmission strategy, and that *V. culicis* would try to react by enhancing its transmission potential.

2.3 Materials and methods

2.3.1 Mosquitoes

Aedes aegypti, the main vector of yellow, dengue and chikungunya fevers, is a ubiquitous mosquito in tropical and subtropical regions. Its ecology, physiology and genetics have been extensively studied (Christophers 1960; Nene et al. 2007). The larvae of *Aedes aegypti* may experience a wide range of growth conditions and environmental fluctuations in either natural or artificial containers (Chan et al. 1971), leading to a developmental period ranging from 6 to 30 days (Southwood et al. 1972). We used the UGAL strain of the mosquito *A. aegypti* provided by Patrick Guérin (University of Neuchâtel). The colony is maintained at standard conditions of 26 °C, 70% humidity and a 12 h: 12 h light:dark photoperiod. In each generation, we keep 1500 adult mosquitoes, which have constant access to a 10% sucrose solution.

2.3.2 Microsporidia

Edhazardia aedis and *Vavraia culicis* are microsporidians, a group of endocellular parasites that are common in insects (Becnel & Andreadis 1999). Both of them are natural parasites of *Aedes aegypti* and were provided by J. J. Becnel (USDA, Gainesville, USA). *E. aedis* is specific to *A. aegypti* (Becnel & Johnson 1993), whereas *Vavraia culicis* can infect several genera of mosquitoes (Weiser & Coluzzi 1972).

Edhazardia aedis has a complex life cycle involving horizontal and vertical transmission (Becnel et al. 1989; Becnel et al. 1995; Johnson et al. 1997; Desjardins et al. 2015). The two transmission routes involve 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 that lays infected eggs. 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. Almost no vertically infected individuals survive beyond pupation, so vertical transmission is almost always followed by horizontal transmission. 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, this results in the next round of vertical transmission. 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 another round of horizontal 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 can be transmitted horizontally when the infected larvae die and release the spores into the water (Agnew et al. 1999; Becnel et al. 2005). They can also be transmitted (horizontally or vertically) by adhering to the eggs of an infected female and thus reaching a new larval site.

2.3.3 Experimental design

The purpose of this study was to see how within-host competition, timing of co-infection and the host's resources affect the mortality of infected mosquitoes and the transmission of each parasite. We therefore ran (and analysed) two experiments, one for each focal parasite and its competitor. Since a main question was about transmission, we did not include uninfected mosquitoes in the experiment.

We treated each species of parasite in a full-factorial design, rearing the mosquito larvae in one of two feeding regimes, exposing them to the parasite at one of two days, and either exposing them to the other species or leaving them as single infections on the other day. We then followed the mosquito's development to evaluate three life-history traits – juvenile mortality (probability of emergence), age at emergence and longevity – and the parasite's development to evaluate the potential for transmission among larvae and from adults to larvae. The experiment was performed in the standard rearing conditions of the colony: 26° C, 70% relative humidity and a 12h light and 12h dark regime.

Colony eggs were rehydrated in 100 mL of deionised water and were then synchronously hatched under partial vacuum. 1200 larvae were haphazardly transferred to and reared individually in 12-well tissue-culture plates filled with 3 mL of deionized water. Each well on a plate represented a different treatment (see below).

We used larval food (standard and low food, i.e. 100% or 50% of a standard det), timing of infection (early and late infection, i.e. two and four days after hatching) and coinfection status (presence or absence of the other parasite) as experimental factors for a total of 12 treatments (Table 1). As we were only interested in the consequences of multiple infections compared to single infection, we did not include a treatment without infection. The high food (HF) regime per larva was at age 0: 0.06 mg of TetraMin™ fish food per larva, age 1 day: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg, 0.32 mg from age 5 onwards. The larvae received 10^4 spores of *V. culicis* (V) in 100 µL, 400 uninucleate spores of *E. aedis* (E) in 100 µL, or 100 µL of a solution of crushed uninfected larvae as control (C) two or four days after hatching in function of their treatment (Table 1). Our earlier studies have shown that these densities of spores assure high infection rates and prevents excessive mortality rates. The inoculate of *V. culicis* was obtained from our stock of spores maintained at 4° C. Vertically infected larvae of *E. aedis* were hatched 7 days before the days of infection and then killed to obtain the uninucleate spores. Twenty larvae were crushed and homogenized in an Eppendorf tube containing 1mL of deionized water. The concentration of the spores was determined with a hemocytometer and a phase contrast microscope (Zeiss Axio Lab.A1) to prepare the infection solution of both parasites. 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 the individuals (larvae, pupae and adults) was checked every 24h 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.

V. culicis and *E. aedis* and the type of spore of *E. aedis* can be differentiated visually. The infection was verified adding 0.1 mL of deionized water to the tubes and homogenizing the samples using the

TissueLyser LT - QIAGEN beads machine. A volume of 8 µL of the obtained solution was placed on a hemocytometer under a phase contrast microscope (Zeiss Axio Lab.A1) to check for the presence of spores and to identify their species and type. The treatments of the samples were unknown while we counted the spores.

2.3.4 Statistical analysis

Following our experimental design, we analysed the two focal parasites separately. We analysed the age at emergence of females with a Cox proportional hazards regression model, the survival of the juveniles with a generalized linear model (glm) with binomial distribution, and female longevity with a Cox proportional hazards regression model censoring the mosquitoes killed on day 24 (the end of the experiment). In each analysis, the co-infection status, time of infection, and food availability were used as factors.

We considered two routes of horizontal transmission of *Vavraia*: among larvae or from females to larvae. We therefore define two measures of transmission: the potential for transmission among larvae was the percentage of dead larvae that harboured spores and the potential of female-to-larva transmission was the percentage of females harbouring spores. Since *Edhazardia* uses two types of spores for its two transmission strategies, we distinguished between the production of the spores and the potential for transmission. For transmission among larvae we thus analysed the percentage of mosquitoes that harboured uninucleate spores and the percentage of mosquitoes that died as juveniles harbouring uninucleate spores, and for vertical transmission we analysed the percentage of mosquitoes that harboured binucleate spores and the percentage of mosquitoes that survived to become adult females harbouring binucleate spores. All transmission measures were considered as the qualitative factors (possible or not possible). We did not estimate a quantitative measure of transmission (number of spores), for in our experiment very many mosquitoes harboured no horizontally transmitted spores (84% of *Vavraia*-exposed; 93% of *Edhazardia*-exposed mosquitoes). We analysed the potential of each transmission route with a glm with binomial distribution, including co-infection status, time of infection, juvenile food availability and their interactions as factors. All statistical analyses were carried out with R (version 3.4.2, R Development Core Team 2013). We used the “survival” package (Therneau 2015) for the Cox model.

Table 1 Experimental treatments

Treatment	Larval Food	Age at infection		Coinfection
		Early Infection	Late Infection	
1	High	*Vavraia*	–	No
2	High	–	*Vavraia*	No
3	High	*Edhazardia*	–	No
4	High	–	*Edhazardia*	No
5	High	*Edhazardia*	*Vavraia*	Yes
6	High	*Vavraia*	*Edhazardia*	Yes
7	Low	*Vavraia*	–	No
8	Low	–	*Vavraia*	No
9	Low	*Edhazardia*	–	No
10	Low	–	*Edhazardia*	No
11	Low	*Edhazardia*	*Vavraia*	Yes
12	Low	*Vavraia*	*Edhazardia*	Yes

2.4 Results

2.4.1 Focal parasite: *Edhazardia aedis*

Juvenile mortality

Food strongly affected juvenile mortality, with 91% (c.i. 89-94) of the well-fed larva and 81% (76-85) of the larvae fed on the low diet surviving to emergence (χ^2=14.4, df=1, p<0.001) (Figure 1). Confection led to greater survival (88%; 85-91) than single infections (84%, 79-87), though the effect was not quite significant (χ^2=2.8, df=1, p=0.092). The three factors – food, timing of infection and coinfection – interacted to determine survival (χ^2=5.6, df=1, p=0.018), with the highest survival to emergence (95%; c.i. 86-98) observed if the mosquitoes had been well fed, infected late with *Edhazardia* and coinfected with *Vavraia* and the lowest survival (68%; 57-77) observed if the mosquitoes had been fed on the low diet, infected early with *Edhazardia* and not coinfected with *Vavraia*. The other factors and interaction were not significant (χ^2<2.5, df=1, p>0.12).

Age at emergence

88% of the surviving juveniles emerged within 10 days after hatching. The percentage emerging by day 10 depended on food (low food 72%, c.i. 64-79; high food 100%, 98-100; χ^2=2273, df=1, p<<0.001) and it depended on coinfection (singly infected: 92%, 87-96; coinfected: 82%, 75-88; χ^2=3.1, df=1, p=0.076), with the effect of coinfection of course only apparent at low food levels (singly infected: 83% (71-91); coinfected: 61% (49-73)) (χ^2=2.9, df=1, p=0.091). The effect of coinfection was also stronger if

mosquitoes had been infected late (singly infected: 96% (89-99); coinfected: 74% (61-83)) than if they had been infected early (88% (77-95) vs. 91% (81-96)) (χ^2=22.3, df=1, p<<0.001). The other interactions had no significant effect (χ^2<1.6, df=1, p>0.21).

Longevity

33% of the mosquitoes survived up to the end of the experiment (24 days after hatching, Figure 2), with well-fed mosquitoes (28%, c.i. 21-36) living less long than mosquitoes reared at low food (38%, 30-47) (χ^2=3.7, df=1, p=0.056). The effect of food was more apparent if the mosquitoes were singly infected (low food: 41% (29-55); high food: 19% (11-30)) than if they were coinfected (35% (24-48) vs. 38% (27-49)) (χ^2=2.7, df=1, p=0.101). If infection was early, fewer mosquitoes (23%, 16-31) survived than if infection was late (43%, 34-51) (χ^2=16.3, df=1, p<<0.001). The other factors and interactions were insignificant (χ^2<1.6, df=1, p>0.208).

Among-larva transmission

Edhazardia produced horizontally transmitted (uninucleate) spores in 7% of the infected mosquitoes (Figure 1). It was more likely to produce them, if the mosquito had been reared at low food (8% of the mosquitoes) than at standard food (5%, 2-9) (χ^2=6.5, df=1, p=0.011) and if it had been infected early (10%, c.i. 5-13) than late (3%) (χ^2=12.2 df=1, p<0.001). The effect of food was less apparent in early infections (10%, 5-18 vs. 9.7%, 4-18) than in late infection (6%, 2-14 vs. 0%, 0-4) (χ^2=6.0, df=1, p>0.014). All other factors and interactions were insignificant (χ^2<0.5, df=1, p>0.484). Only two mosquitoes that died before emergence also harboured uninucleate spores and would thus be able to transmit the parasite to other larvae. While a statistical analysis is meaningless, it should be noted that both of these individuals had been fed on the low diet and infected early; one of them was singly infected, the other coinfected.

Vertical transmission

52% of the infected mosquitoes carried vertically transmitted, binucleate spores (Figure 3). *Edhazardia* was more likely to produce these, if it had been infected early (60% of the mosquitoes, c.i. 53-68) than late (43%, 36-51) (χ^2=9.7, df=1, p=0.002), and if it was singly infected (57%, 49-64) than if it was coinfected (47%, 40-55) (though this difference was not quite significant: χ^2=2.8, df=1, p<0.095). The likelihood that is transmitted vertically (that is produces binucleate spores in adult females) was affected only by the food level, with standard food giving a higher potential for vertical transmission (52% of the mosquitoes, c.i. 44-60) than low food (31%, 25-39) (χ^2=8.8, df=1, p=0.003). No other factors were significant (χ^2<1.8, df=1, p>0.186).

2.4.2 Focal parasite: *Vavraia culicis*

Juvenile mortality

In the high and low food treatment 94% (95% c.i: 92%-97%) and 87% (83-90) survived as juveniles, respectively (χ^2=10.1, df=1, p=0.002) (Figure 1). This effect of food was more apparent if mosquitoes had been infected by *Vavraia* early than if they had been infected late: after an early infection giving the larvae less food decreased survival from 96% (92-98) to 84% (78-89), while after a late infection survival decreased from 93% (89-96) to 90% (85-94) (interaction food * time of infection: χ^2=3.5, df=1, p=0.062). Coinfection with *Edhazardia* decreased survival from 93% (90-95) to 88% (85-91), though this difference was not statistically significant (χ^2=3.1, df=1, p=0.077). The other factors and interactions were far from significant (χ^2<0.9, df=1, p>0.344).

Age at emergence

81% of the surviving juveniles emerged within 10 days after hatching. The percentage emerging by day 10 depended on food (low food 60% (c.i.: 52-69%), high food 100% (97-100); χ^2=237, df=1, p<<0.001) and on the time of infection (early infection: 72% (64-79), late infection: 89% (83-94); χ^2=28.9, df=1, p<<0.001). Since at high food all mosquitoes emerged by day 10, the effect of time of infection was only apparent at low food (early infection: 35% (23-48), late infection: 80% (70-89); interaction food * time of infection χ^2=5.1, df=1, p=0.024). The other factors and interactions had no significant effect (χ^2<0.4, df=1, p>0.544).

Longevity

51% of the mosquitoes survived up to the end of the experiment (24 days after hatching, Figure 2). If infection was early, more mosquitoes (60%; c.i. 51-68) survived than if infection was late (43%; c.i. 35-52) (χ^2=6.6, df=1, p=0.010), and if mosquitoes were coinfected, they were less likely to survive (37%; c.i. 29-45) than if they were infected only by *Vavraia* (67%; c.i. 58-74) (χ^2=23.5, df=1, p<<0.001). While the effect of the timing of infection by *Vavraia* was more pronounced for coinfected mosquitoes (early: 50%, late: 24%) than for singly infected ones (early: 68%, late: 64%), the interaction between timing and co-infection was not significant (χ^2=2.1, df=1, p=0.15). The other factors and interactions were insignificant (χ^2<1.5, df=1, p>0.23).

Among-larva transmission

4% of the *Vavraia*-infected mosquitoes died before emergence harbouring spores and could have transmitted the parasite to other larvae (Figure 1). The potential for transmission of *Vavraia* among larvae was higher if the mosquito had been fed on the low diet (6%; c.i.: 4-9) than if they had received the standard diet (1%; c.i.: 0-3) (χ^2=10.2, df=1, p=0.001) or if it had coinfected with *Edhazardia* (5.4%) than if it had been infected only with *Vavraia* (2.2%) (χ^2=11.8, df=1, p<0.001). Early infection led to greater potential for among-larvae transmission (2%; 1-4%) than late infection (5%; 3-8%) (χ^2=5.2, df=1,

p=0.022). Timing of infection and coinfection interacted, so that the potential for among-larvae transmission ranged from 0% (c.i. 0-2) for late, singly infections to 7% (4-12) for early infections co-infected by *Edhazardia* (χ^2=4.4, df=1, p=0.037). The other interactions were insignificant (χ^2<1.8, df=1, p>0.175).

Adult to larva transmission

12% of the mosquitoes survived to emerge as adult females and carried spores (Figure 3). Early infection led to greater potential for transmission from adult females to larvae (18%; 15-23%) than late infection (6%; 4-9%) (χ^2=30.9, df=1, p<<0.001). The effect of timing of infection depended on the level of food; while at low food, late infection halved the potential for transmission (from 17% to 8%), at standard food transmission was decreased fivefold (from 20% to 4%) (χ^2=4.5, df=1, p=0.033). Transmission from adults to larvae was higher in singly infected individuals (14%; c.i. 10-18) than in coinfected ones (11%; c.i.=8-14) (χ^2=4.57, df=1, p=0.031). The effects of timing of *Vavraia*-infection and co-infection by *Edhazardia* affected transmission: while after early infection, co-infection had little impact (co-infected mosquitoes: 18%; singly infected mosquitoes: 19%), there was a greater than two-fold decrease due to co-infection after late infection (4% vs. 9%) (χ^2=3.5, df=1, p=0.063). The other factors and interactions were insignificant (χ^2<2.6, df=1, p>0.109).

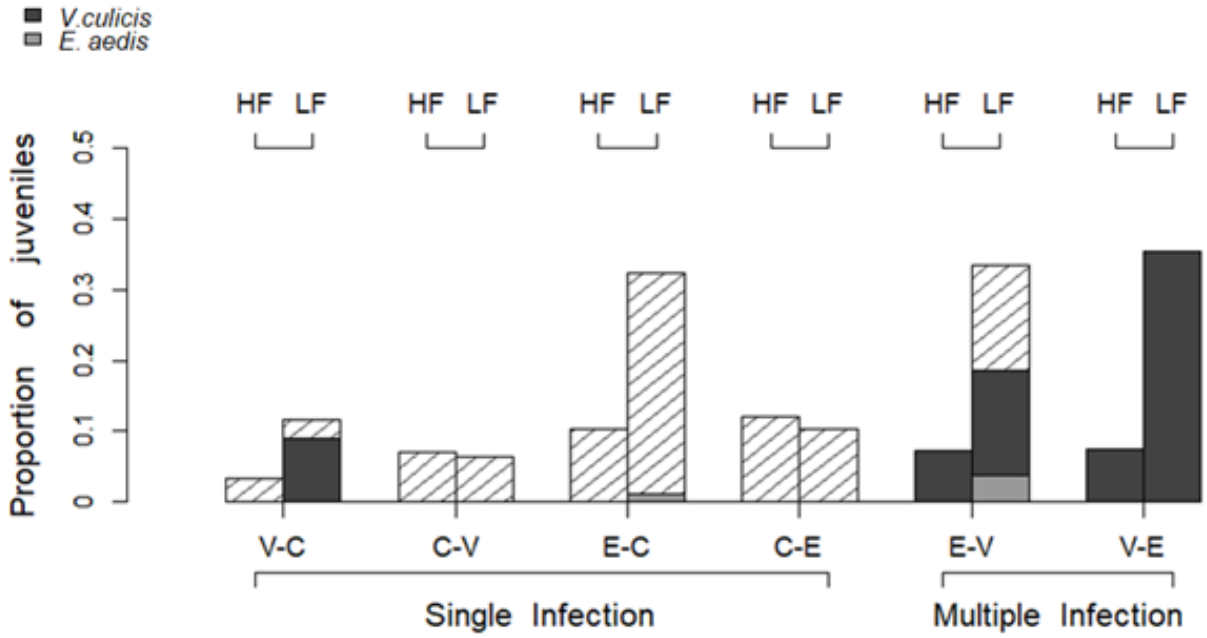

Figure 1 The proportion of individuals dying as juveniles (dashed bars), and of those the proportion harbouring spores and affording the potential for horizontal transmission for *Edhazardia aedis* (light grey) and *Vavraia culicis* (dark grey). HF and LF represents respectively the high and low food regime. The parasite treatment and the order of infection are given by the combination of two letters marked as V = *V. culicis*, E = *E. aedis* and C = Control solution (no parasite). The first letter of the label on the x axis represent the early infection (day 2) and the second letter the late infection (day 4). E.g. E-V = *E.aedis* early infection (day 2) and *V. culicis* late infection (day 4).

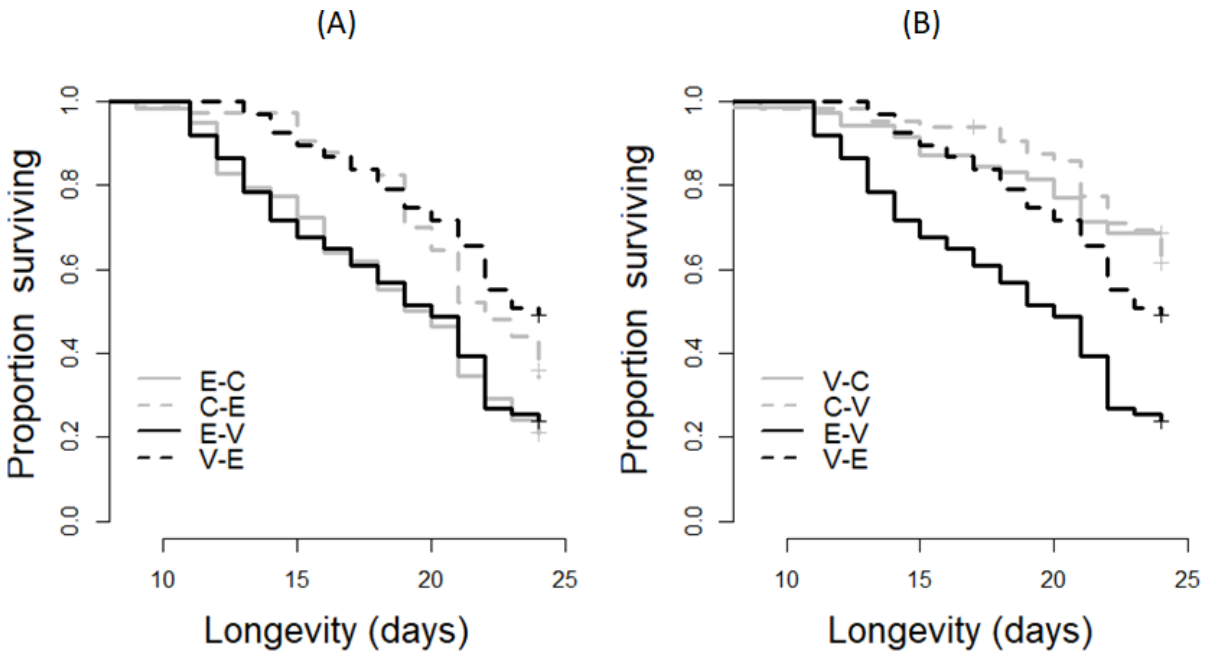

Figure 2 Proportion of surviving females in function of their longevity after emergence for *Edhazardia aedis* (A) and *Vavraia culicis* (B) in single (grey lines) or multiple infection (black lines) at early (continuous lines) or late (dashed lines) stage of development. The black lines represent the survival curves of the multiple infections and are the same in (A) and (B).

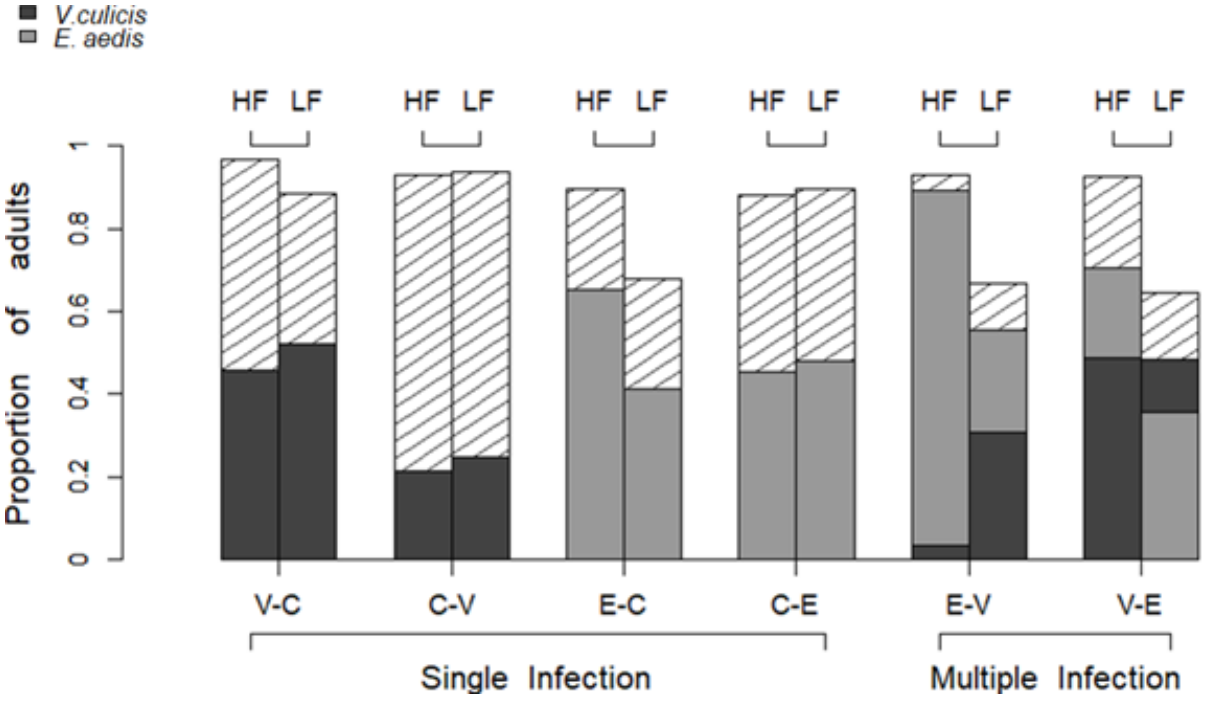

Figure 3 The proportion of individuals emerging (dashed bars), and of those the proportion harbouring spores and affording the potential for vertical transmission for *Edhazardia aedis* (light grey) and horizontal transmission of *Vavraia culicis* (dark grey). The HF and LF bars represent respectively the high and low food regime. The combination of two letters marked as V = *V. culicis*, E = *E. aedis* and C = control solution (no parasite) represents the parasite treatment and the order of infection. E.g. E-V = *E.aedis* early infection (day 2) and *V. culicis* late infection (day 4).

2.5 Discussion

Overall, the interactions between co-infection, the order of infection and host's resources, highly affected parasites' transmission, virulence, and the life-history traits of the host. Here we describe the results meaningful to our expectations.

In our study the reduced availability of resources slowed host's growth, delayed age at emergence and increased the probability of dying in the water for the juveniles carrying single infection with either *E. aedis* or *V. culicis*. Together with the low food, early infection and co-infection further delayed age at emergence and increased juvenile mortality favouring the exploitation by the parasite, the transmission among larvae (horizontal transmission) and thus virulence.

The potential for horizontal transmission of *E. aedis* was higher when infecting first and in low food conditions. This could be a strong phenomenon of competitive exclusion realized by *V. culicis* but this is difficult to evaluate giving the minimal number of individuals found harbouring uninucleate spores. Nonetheless, the overall higher mortality rate in low food treatment and the delayed age at emergence of *E. aedis* suggests an investment in horizontal transmission under slow growth conditions of the host, which was in line with our predictions and with previous studies (Koella et al. 1998; Agnew & Koella 1999).

In multiple infection, the probability of horizontal transmission considerably increased for *V. culicis*. For the juveniles harbouring a co-infection, in particular at low food conditions, were more likely to die sooner as juveniles (high virulence), *V. culicis* had an advantage by increasing the probability of horizontal transmission, securing this way some potential transmission among larvae. In accordance with our expectation, being the first parasite to infect the juveniles increased the probability to overcome the competitor and transmit horizontally. It is likely that *V. culicis,* by infecting first, was able to fully exploit host's resources in accordance with the accumulation of spores in the juveniles over the time spent in the aquatic environment (Bedhomme et al. 2004). The result confirms previous findings where *V. culicis* anticipated the production of the spores in multiple infections with either *E. aedis* (Duncan et al. 2015) or *Ascogregarina culicis* (Fellous & Koella 2009). This is also consistent with a shift in life history to an earlier investment in reproduction under detrimental conditions (Stearns & Koella 1986), represented here by competition and low availability of resources for the host. Thus, *V. culicis* reacted accordingly to the harsh environment and maximized its early investment in horizontal transmission.

The high food conditions and the late infections increased the probability of emergence and lead to a faster development into adults in accordance to our expectations (Koella et al. 1998; Agnew & Koella 1999). In the transmission from adults to larvae, we found an asymmetric competitive relationship

between the respectively vertical and horizontal transmission of *E. aedis* and *V. culicis*. The transmission potential of the focal parasite was affected by the time of infection and the resources during the juvenile stage of the host, but for *V. culicis* it further depended on the presence of the competitor.

The vertical transmission of *E. aedis* is only possible via infected females laying eggs, thus the high probability of finding binucleate spores in the females reared at high food was expected (Agnew & Koella 1999). If host's growth is fast due to favourable nutritional conditions, the probability of emergence into adults are higher, and the parasite may expect higher fecundity and invests more in vertical transmission. In addition, *E. aedis* increased its probability of vertical transmission with early infections. Infecting first, it had more time to growth and pass from the horizontally transmitted uninucleate spores to vertically transmitted binucleate spores following its fixed stages of development. Besides, competition was not reducing the capacity of vertical transmission, whereas this was the case with early infections. This suggests that the transmission from adult to larvae was consistently efficient no matter the presence of *V. culicis*.

The transmission strategy of *V. culicis* from adults to larvae differs from *E. aedis*, it remains strictly horizontal and requires the adults to die in the water in order to release the spores and infect new larvae. The probability of infection of *V. culicis* in the adults increased when infecting first in single infection and multiple infection. In contrast, when *E. aedis* was already present (*V. culicis* late infection), it almost excluded and drastically reduced the strength of infection of *V. culicis*. Both microsporidia enter the host through the same intestinal cells, it is possible that *E. aedis* impedes the second invader to infect and spread through host cells (competitive exclusion). Many examples exist showing how vertical transmitted parasites may protect their host against other pathogens (Oliver et al. 2003; Ferrari et al. 2004; Hedges et al. 2008; Jaenike et al. 2010), as these parasites and the host share to a large degree their evolutionary interests (Frank 1996). The success of the vertical transmitted parasites indeed, is coupled with the lifetime reproductive success of the host (Frank 1996).

However, during co-infection, the vertical transmission component of *E. aedis* appears to partially match host's interests, for it seems that the damages to the host are due to *E. aedis* and not to *V. culicis*. Indeed, the longevity of the mosquitoes infected with *E. aedis* in single or multiple infection did not differ, in contrast, when considering *V. culicis* as the focal parasite, co-infection reduced adult's longevity and virulence was higher, suggesting that *E. aedis* was driving adult's mortality. Moreover, *V. culicis* did not increase its transmission potential during co-infection despite the transmission from adult to larvae requires the death of host, which was enhanced by the presence of *E. aedis*. The effect is probably due to specificity of *E. aedis* and to the natural virulent component of its horizontal

transmission with the production of uninucleate spores (Agnew & Koella 1997). In line with our predictions, the results show that the vertical transmitted stage of *E. aedis* is limiting the competitor's growth, and further suggest how it might be winning the competition in the adults. For *E. aedis*, the higher mortality in the adults might be of relative importance, as only few spores are necessary to infected almost all the eggs (Becnel et al. 1995) and consequently a single clutch of eggs can assure a successful vertical transmission.

Here, we highlighted that the time of infection is a fundamental factor affecting multiple infections as recently modelled (Marchetto & Power 2018), with early infections resulting in higher transmission potential. This is also compatible with the idea of parasites competing for common resources, where the first parasite to invade the host disposes of more nutrients to develop (Alizon et al. 2013). Accordingly, the increased mortality of host with co-infections is likely due to the exploitation of shared resources, thus resulting in higher virulence for this system. However, the relationship between the transmission and the virulence of the two parasites differ in the juvenile and adult stage of the host, which in turn was affected by the availability of resources. The horizontal transmitted parasite *V. culicis* reached higher transmission and won the competition over the mixed-mode transmission parasite *E. aedis* during the juvenile phase of the host. In the adult mosquitoes, the conflicting transmission strategy lead to an asymmetric relationship. When infecting first, the vertical component of *E. aedis* outcompeted the horizontal transmission of *V. culicis* despite the reduced longevity of the adult and the increased virulence should advantage the latter. In this study, differently from what was previously observed (de Roode et al. 2005a; Ben-Ami et al. 2011), we show that co-infection and the interspecific within-host competition may increase the potential transmission of the parasites. The transmission potential of both parasites significantly changes when comparing our sequential order of infection to a simultaneous co-infection in the similar experiment of Duncan et al. (2015). It is difficult to evaluate the different and common points of the studies because, apart from the simultaneous infection, also the inoculation dose, the time of exposure to the parasite during the juvenile stage and the food quantity differ.

In conclusion, multiple infections are widespread and require more attention as many factors, from the order of infection to the conflicting transmission strategy, change and alter within-host competition. Since these interactions are influencing parasite's virulence and transmission leading to complex and unexpected epidemiological outcomes, it is important to investigate the selective pressure acting on them to have a better comprehension of host-parasite evolution.

Chapter 3

Host genotype and environment affect the trade-off between horizontal and vertical transmission of the parasite *Edhazardia aedis*

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3.1 Abstract

If a parasite is able to transmit horizontally or vertically, which transmission mode will it choose? We investigated how the growth conditions and the genotype of the mosquito Aedes aegypti affect the transmission mode of the parasite Edhazardia aedis.

In poor conditions the parasites were more likely to be transmitted horizontally, whereas in favourable conditions they were more likely to be transmitted vertically. Unfavourable conditions delayed emergence, giving the parasite more time to produce its horizontally transmitted stage; in more favourable conditions mosquitoes have greater reproductive success, increasing the effectiveness of vertical transmission. In addition, the parasite's ability to transmit vertically was influenced by the genetic background of the host (i.e., its full-sib family), giving a genetic correlation between the host's life-history and which of the parasite's transmission mode it enables. In particular, genotypes with large bodies (and therefore high fecundity) were more likely to enable vertical transmission than genotypes with small bodies. This led to a trade-off among the host's families (which can be interpreted as a genetic correlation) for the parasite's transmission mode. Since horizontal transmission is linked to higher virulence than vertical transmission, the host's contribution to transmission mode has important consequences for the evolution of parasites with mixed-mode transmission.

3.2 Introduction

The rate of transmission and the severity of symptoms (i.e. virulence) determine a parasite's damage to a population of hosts, and they are the center of most modern ideas about the evolution of parasites (Gandon et al. 2001; Leggett et al. 2013). Ideas about virulence, for example, are often based on a link between virulence and rate of transmission, and explain virulence as an unavoidable side-product of selection for more transmission (Alizon et al. 2009). Virulence is also predicted to change according to the mode of transmission. Horizontal transmission can be associated with high virulence, if the strong exploitation of the host increases the rate of transmission. In contrast vertical transmission is expected to be associated with low virulence, for the parasite and the host share to a large degree their evolutionary interests (Frank 1996).

While the role of transmission mode on the evolution of virulence is often studied, relatively little attention has been given to how transmission mode evolves (Ebert 2013; Antonovics 2017). Theoretical exceptions predict the conditions that enable vertical and horizontal transmission to coexist and their resulting levels of virulence (Lipsitch et al. 1996; Ferdyn & Godelle 2005). These models can be applied to many parasites with mixed modes of transmission such as the medically important viruses HIV, human papilloma virus, and hepatitis B and C viruses.

In many parasites with such mixed-mode transmission, the two transmission modes are physiologically, developmentally or evolutionarily linked (Ebert & Mangin 1997; Messenger et al. 1999; Magalon et al. 2010; Dusi et al. 2015). In many cases, this link means that transmitting with one mode, either vertically or horizontally, reduces transmission with the other (Antonovics 2017), but vertical and horizontal transmission can also be positively correlated (de Roode et al. 2009; Kover & Clay 1998; van Frankenhuyzen et al. 2007). When a trade-off between the two transmission modes is present, it strongly influences the parasite's evolution and dynamics (Lipsitch et al. 1995; Lipsitch et al. 1996). For example, the nature of virulence of the horizontal component (additional mortality or reduced fecundity in the host) may impose constraints on the shape of the trade-off. Only a convex shape of the trade-off will allow the coexistence of the two transmission modes, while a concave one will lead to the fixation of either one or the other (Ferdyn & Godelle 2005). This implies that the trade-off between the two transmission modes is an important part of a parasite's epidemiology (van Baalen 2000).

In most cases we know little about what controls this trade-off, and in particular about the genetic basis of the trade-off. Although several studies have measured the trade-off between horizontal and vertical transmission, almost all of them have focused on the variation due to the environment rather than to the host's or parasite's genotypes. One example is the bacterium *Holospora undulata* that

infects the ciliate *Paramecium caudatum*. The bacterium has a reproductive stage that is used to infect the daughter nuclei of infected cells, and it has an infectious stage that can be transmitted horizontally. If the system is maintained in a situation enabling high replication rates of the host, the parasite remains mainly in the reproductive stage and is passed on vertically to the daughter cells of mitotically dividing paramecia; in contrast, at low population growth rate, the parasite differentiates into infectious forms and is transmitted horizontally (Kaltz & Koella 2003). A second example is the microsporidian *Edhazardia aedis* that infects the mosquito *Aedes aegypti*. The parasite has two forms of spores: binucleate spores that infect the eggs of infected females and are transmitted vertically, and uninucleate spores that can kill infected larvae and are transmitted horizontally among larvae. There is an obvious trade-off in this system: horizontal transmission requires the death of larvae, so that it precludes vertical transmission. The investment in the two spore types is regulated in a way that horizontal transmission becomes more likely as food conditions deteriorate and therefore larvae grow more slowly (Koella et al. 1998; Agnew & Koella 1999). An adaptive explanation is that only good food conditions enable the host to lay many eggs and therefore the parasite to have efficient vertical transmission; the potential for vertical transmission decreases as larval growth slows, making the mosquitoes more attractive to exploitation for horizontal transmission (Koella et al. 1998; Agnew & Koella 1999).

Such an adaptive explanation requires that the transmission mode is ultimately controlled by the parasite's genes. Parasitic control has indeed been shown in a number of cases. Thus, differences among strains of the fungus *Atkinsonella hypoxylon* differ in the production of the fruiting bodies responsible for horizontal transmission (Kover & Clay 1998). Moreover, manipulating levels of horizontal and vertical transmission have led to evolutionary changes of the pathogen's transmission mode in several studies, for example bacteriophages (Bull et al. 1991), barley stripe virus infecting *Hordeum vulgare* (Stewart et al. 2005), and the bacterium *Holospora undulata* infecting *Paramecium caudatum* (Magalon et al. 2010). While there are thus a few suggestions of the parasite's genes control of its transmission mode, we may expect that the transmission mode and the trade-off between horizontal and vertical transmission are also influenced by the host's genes. Thus, selection for delayed pupation of the mosquito brings with it more vertical and less horizontal transmission of *E. aedis*. (Agnew & Koella 1999). Furthermore, several traits of host-parasite interactions are governed by a combination of host and parasite genotypes (Casadevall & Pirofski 2003; Lambrechts et al. 2006; Refardt & Rainey 2010). The interaction between host and parasite genotypes govern, for example, the variability of the virulence and transmission of *Holospora undulata* (Restif & Kaltz 2006), and the production of the fruiting bodies of the fungus *Epichloë elymi* depends on the genotype of the grass *Elymus hystrix* on which the fungus is growing (Tintjer et al. 2008). A different genetic background of a parasite's host may therefore promote divergent evolutionary trajectories for the parasite, for each

host will cause a particular selection pressure on the parasite. Parasites infecting populations of hosts with different genetic background may therefore experience different selection pressures. Such a host-parasite interaction may consequently shape spatial gradients of local adaptation in accordance with the idea of the geographic mosaic of co-evolution (Thompson 1999; Thompson 2006).

In this study, we investigated how the host's genotype and food environment interact to influence the horizontal and vertical transmission of *E. aedis.* We expected that lower food availability would favour horizontal over vertical transmission. We further expected that the mosquito's genotypes enabling large size and long life would favour vertical transmission, while genotypes with a long development would favour horizontal transmission, and that these associations would be more apparent when food is scarce. Finally, we expected to find a trade-off in the host's potential to transmit the parasite horizontally or vertically.

3.3 Material and methods

3.3.1 Mosquitoes

Aedes aegypti, a mosquito that occurs throughout the tropics and subtropics, is the main vector of yellow, dengue and chikungunya fevers. Its physiology, genetics, and ecology have been intensively studied (Christophers 1960; Nene et al. 2007). The larvae of *Ae. aegypti* grow in natural or artificial containers and their development depends on many environmental factors (Chan et al. 1971). Two aspects that make it suitable for lab work is that its eggs can be stored for a long period and that a single mating lets the female lay eggs throughout her life (Munstermann 1997; Oliva et al. 2014). We used the UGAL strain of the mosquito *Ae. aegypti* (obtained from P. Guérin, University of Neuchâtel), an undocumented strain that was established in the 1970s (Kuno 2010). The mosquitoes were maintained at 26 °C, 70% humidity, 12 h:12 h light:dark photoperiod.

3.3.2 Microsporidia

Edhazardia aedis was provided by J. J. Becnel at the United States Department of Agriculture (Gainesville, Florida). This microsporidium, a specific parasite of *Ae. aegypti*, has a complex life cycle (Figure 1) involving horizontal and vertical transmission (Becnel et al. 1989; Nasci et al. 1992; Becnel et al. 1995; Johnson et al. 1997; Desjardin et al. 2015) and using two types of morphologically distinguishable spores: uninucleate spores for horizontal transmission and binucleate spores for vertical transmission. Mosquito larvae are infected when they ingest uninucleate spores suspended in water. After a period of development, the parasite produces binucleate spores in adult females; these are responsible for vertical (transovarial) transmission. Males provide no opportunity for vertical transmission, so adult males are a dead-end for the parasite. Once the infected eggs hatch the parasite develops into uninucleate spores. These vertically infected mosquitoes die as juveniles and release the

uninucleate spores into the water. These uninucleate spores are eaten by larvae to complete the life-cycle. Thus, larvae that acquire the spores horizontally usually go on to transmit vertically, while larvae that are infected vertically die as larvae. Occasionally, as illustrated in Figure 1, horizontally infected larvae die before emergence, which can result in a second round of horizontal transmission without the requirement of vertical transmission (Becnel et al. 1989). Note that underlying this sequence of transmission is a fixed developmental sequence (though not necessarily fixed timing) of the parasite that alternates the production of binucleate and uninucleate spores (Becnel et al. 1989; Johson et al. 1997). Thus, if the horizontally infected individuals die for a second round of horizontal transmission, the parasite has switched from uninucleate to binucleate and then back to uninucleate spores.

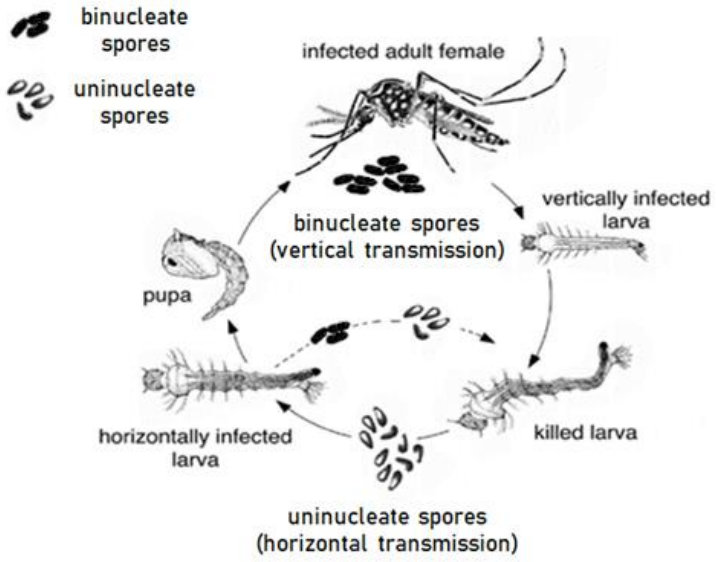

Figure 1 Life cycle of the microsporidia *Edhazardia aedis* infecting the mosquito *Aedes aegypti*. Usually (solid line), the parasite alternate vertical and horizontal transmission using two types of spores. Repeated horizontal transmission is possible (dashed line). Since the parasite's life-cycle involves a strict alternation of binucleate and uninucleate spores, repeated horizontal transmission implies that the parasite goes through its complete developmental sequence – producing first binucleate and the uninucleate spores – within a juvenile mosquito. (Modified from Koella & Agnew 1999).

3.3.3 Experimental design

Our main goal was to see how the host's genotype and environment affect the developmental trade-off between horizontal and vertical transmission. We approximated genetic variation with the variation among full-sib families, where we minimized the maternal effects by rearing the mosquitoes of the parental generation individually in identical environments. We chose as environmental factor the amount of food given to the larvae. We exposed the larvae to a standard concentration of uninucleate spores, and then followed the mosquito and parasite's development to evaluate the potential for horizontal and vertical transmission.

Full sib families

In order to synchronize the hatching and thus obtain larvae of the same age, we hatched uninfected eggs from the colony at low air pressure. The larvae were moved to and individually reared in 12-well tissue-culture plates filled with 3 mL of deionized water. They were fed daily with TetraMin™ fish food (age 0 (day of hatching, day 0): 0.06 mg/larva, age 1: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg, from age 5 onwards: 0.32 mg). Each pupa was placed into a 180-mL plastic cup covered with bed-netting. Three days after emergence, two males were transferred into each of 100 cups all containing one female. Two days later, the males were removed, the females were allowed to blood feed on GZ's arms for 10 minutes, and then given the opportunity to lay eggs on a filter paper for 5 days. The filter paper with the eggs was then stored in a petri dish at the same laboratory conditions as the colony. The cycle of blood-feeding and egg-collecting was repeated for 10 weeks. During this period, adults were also provided with a cotton ball soaked with 10% sugar solution, which was changed every two days. All the eggs collected from one female during her lifespan represented a full-sib family (since females only mate with one male), for a total of 100 full-sib families.

Main experiment

The eggs were gently brushed into petri dishes and rehydrated in deionised water. Thirty of hundred families were synchronously hatched under partial vacuum, twelve of the families with enough larvae were haphazardly chosen for the experiment. The larvae were reared individually in 12-well tissue-culture plates filled with 3 mL of deionized. Each plate contained an individual from each family. Each family experienced two larval food treatments (50% or 100% of our standardized ration of TetraMin Baby™ fish food (see above)). Seventy-two hours after hatching, the larvae were exposed in their wells to 500 uninucleate spores. The spores had been harvested from vertically infected larvae hatched 7 days earlier than the day of infection. Twenty of these vertically infected larvae were crushed and homogenized in an Eppendorf tube adding 1mL of deionized water, and the concentration of the uninucleate spores was determined with a hemocytometer and a phase-contrast microscope (Zeiss Axio Lab.A1). Pupae were individually transferred to Falcon tubes, and the emerging adults were provided with a cotton ball soaked with 10% sugar solution that was changed every 6 days. The survival was checked daily. The dead larvae, pupae and adults were stored in 2 mL plastic tubes at -20°C until further investigation. We measured the spore load for each of the collected individuals after adding 0.1 mL of deionized water and homogenizing the samples with a TissueLyser LT - QIAGEN. The numbers of uninucleate and binucleate spores in the obtained solution were counted with a hemocytometer placed under a phase contrast microscope (Zeiss Axio Lab.A1). The treatment conditions and the family origin of the samples were unknown during the counting of the spores, a total amount of 1904 individuals were used for the analysis with a median of 155 individuals per family.

3.3.4 Statistical analysis

We considered that mosquitoes enabled horizontal transmission, if they died before emerging and harbored uninucleate spores, and that they did not enable horizontal transmission either if they carried no uninucleate spores or did not die as juveniles. We considered that mosquitoes enabled vertical transmission, if adult females carried binucleate spores, and that they did not enable vertical transmission otherwise. Note that we used these qualitative measures of transmission rather than quantitative measures involving the number of spores for two reasons. First it is not known how the number of spores relates to transmission. Second, most of the mosquitoes do not carry any spores (in particular uninucleate spores), so that any quantitative measure would strongly resemble the qualitative one.

We first evaluated the effects of food, full-sib family and their interaction on each transmission mode with a generalized linear mixed effect model with binomial distribution and on age at pupation, wing length and longevity with an ANOVA. For each trait we considered food treatment and family as fixed factors. If the interaction was not significant, we show the results of the analysis that includes only the main effects. Note that we considered family as fixed rather than random, because we were not interested in the variation among families within our colony. Rather, we wanted to check for differences among the families of our study that we could then analyse in the next step of the analysis

Genetic correlation and trade-off

The potential genetic correlation between transmission success (proportion of individuals enabling horizontal or vertical transmission) and life-history traits was analysed with regressions of the family means. We looked at the correlation with juvenile developmental period (days spent in the aquatic environment before emergence as an adult), adult size (wing length), and longevity after emergence in the different food treatments. We investigated a possible trade-off between vertical and horizontal transmission with a linear regression with the family-means of the horizontal and vertical transmission at each food condition. Finally, we evaluated the correlation of vertical transmission of the families between the two food regimes with a linear model. (The analogous analysis for horizontal transmission would be meaningless, for almost all families allowed no horizontal transmission in the good-food environment). All the statistical analyses were performed with R version 3.4.0.

3.4 Results

3.4.1 Effects of food and family on transmission

Families ranged from 11% survival up to emergence (at low food) to 97% (at high food). The effect of food was highly significant for the potential horizontal and vertical transmission. Although 9 % of the mosquitoes that fed the high-food diet and 76 % of the low-food mosquitoes died before emerging, only 2% and 6% of these, respectively, harboured uninucleate spores. The potential for horizontal transmission (the proportion of mosquitoes that died before emergence and harboured uninucleate spores) at high and low food was thus 0.2% and 5% of the exposed individuals (χ^2=54.1, df=1, p<0.001) (Figure 2). Among the mosquitoes that emerged as adult females, 91% and 95% harboured binucleate spores at high and low food, and respectively 42% and 7% of these females enabled vertical transmission (χ^2=350, df=1, p<0.001) (Figure 2).

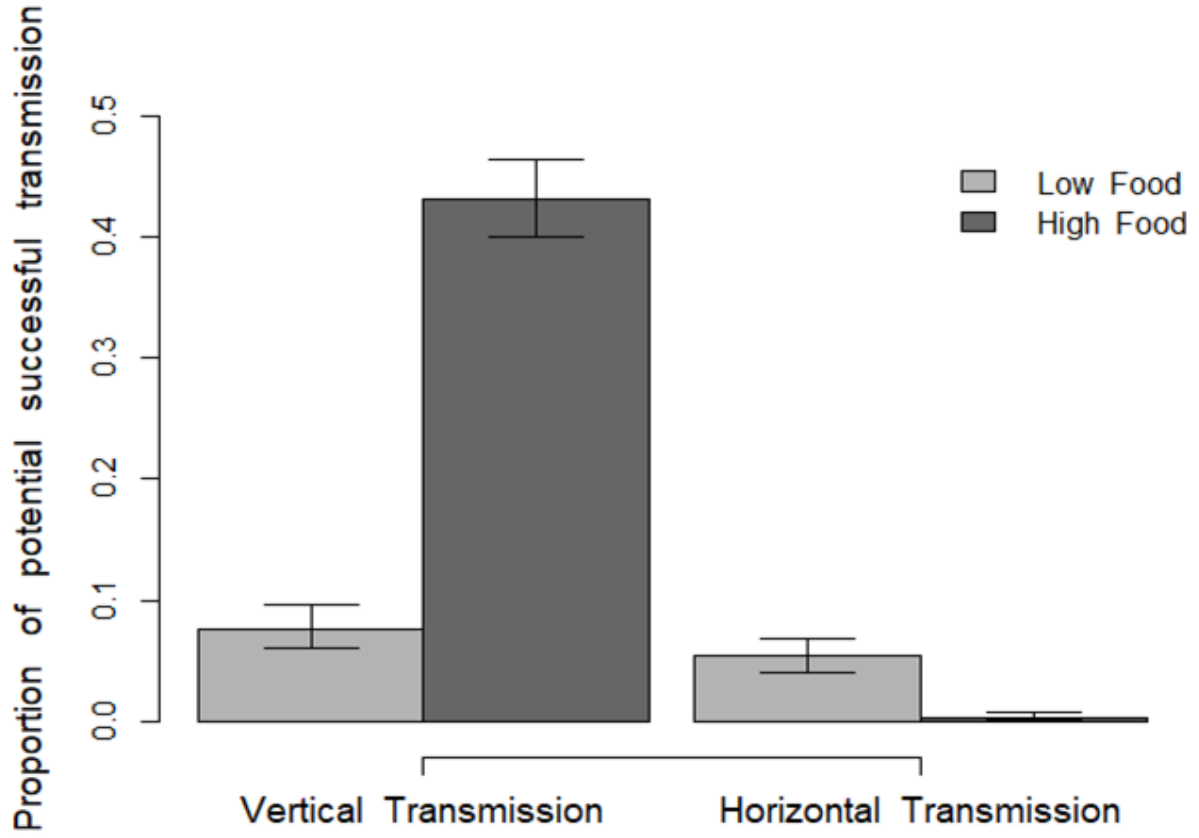

Figure 2 Proportion of potential for vertical and horizontal transmission for low and high food conditions. The bars represent the proportion (± 95 % confidence intervals) of the individuals enabling horizontal or vertical transmission according to their developmental stage (adults harbouring binucleates spores for vertical and juvenile harbouring uninucleate spores for horizontal transmission).

Families ranged from 0% (at high food) to 15% (at low food) horizontal transmission and from 1% (at low food) to 54% (at high food) vertical transmission. The effect of the full-sib families was significant for both the vertical (χ^2=29.4, df=11, p=0.002) and the horizontal transmission mode (χ^2=33.5, df=11,

p<0.001). The interactions between food and family (so, whether the effect of food on the success of the two transmission modes depended on the full-sib families) was significant for vertical transmission (χ^2=25.7, df=11, p=0.007) but not for horizontal transmission (χ^2=5.6, df=11, p=0.90).

3.4.2 Effects of food and family on life history traits

Average age at emergence ranged from 6.2 for one of the families reared with high food to 9.7 days, with highly significant effects of family ($F_{11,990}$=28.3, p<0.001) and food level ($F_{1,990}$=222, p<0.001). Average wing length ranged from 2.82 mm at high food to 2.32 mm at low food (family: $F_{11,990}$=3.34, p<0.001; food: $F_{1,990}$=432, p<0.001). Average longevity after emergence ranged from 12 days at high food to 0.6 days at low food (family: $F_{11,990}$=2.08, p=0.019; food: $F_{1,990}$=160, p<0.001).

3.4.3 Genetic correlations of transmission and life-history

The proportion of the family that enabled horizontal transmission (proportion of individuals within the family enabling horizontal transmission) was higher in low food than in high food conditions (F=35.5, p<0.001) and increased with the average juvenile developmental period of the family (F=36.5, p<0.001), as shown in Figure 3. There was a tendency for the relationship between the family's potential for horizontal transmission and juvenile development to be affected by food (food* juvenile development: F=3.51, p=0.098). Neither body size (F=1.4, p=0.27), longevity (F=0.4, p=0.556) or other interactions (all p-values > 0.13) affected the family's potential for horizontal transmission.

The proportion of the family that enables vertical transmission (proportion of individuals within the family enabling vertical transmission) was higher in high food than in low food conditions (F=96.8, p<0.001) (Figure 4). It increased with mean body size (F=29.8, p<0.001) and longevity (F=5.3, p=0.049) and it decreased with juvenile developmental period (F=8.1, p=0.022). Food modified the effect of body size (food*body size: F= 13.5, p=0.006) and the interaction between mean juvenile development and longevity (juvenile developmental period*longevity: F=5.8, p=0.043; food*juvenile developmental period*longevity: F=6.5, p=0.033). In addition, there was a tendency for an interaction between mean body size and mean juvenile development to affect a family's potential for vertical transmission (F=4.3, p=0.071), but no other interactions were significant (all p-values > 0.28).

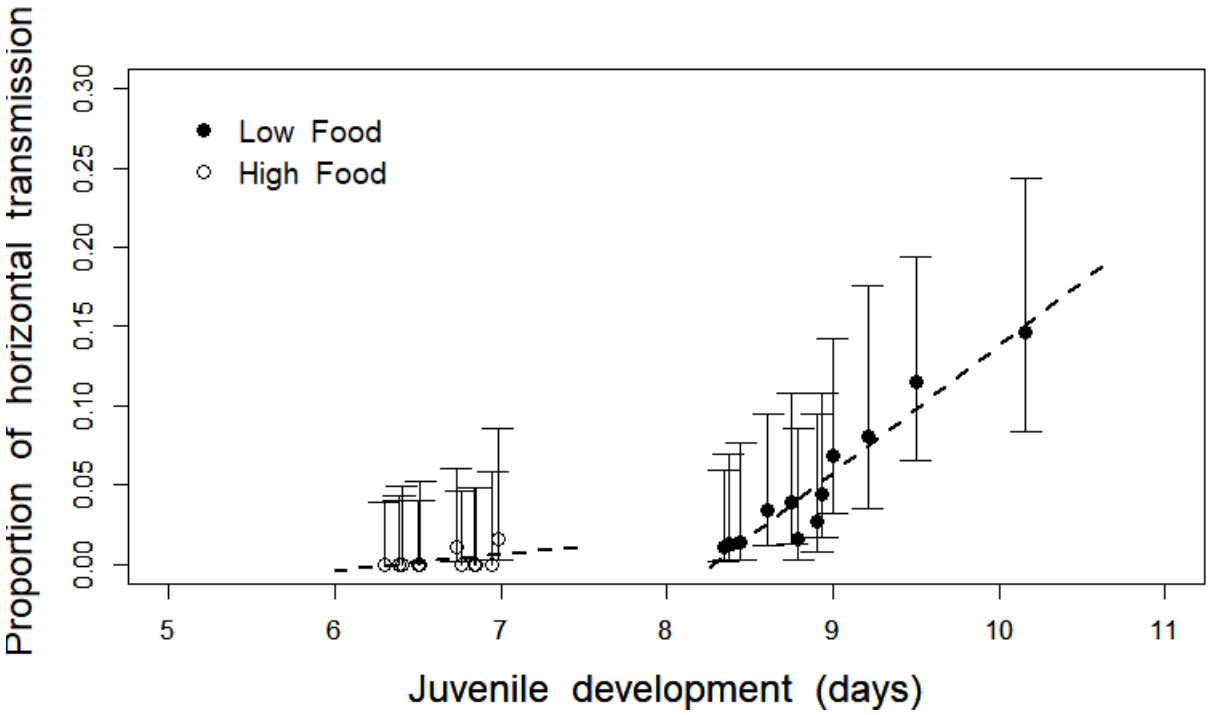

Figure 3 Relationship between horizontal transmission and juvenile development. Dots represent the proportion of horizontal transmission per family (proportion of individuals within the family enabling horizontal transmission) as a function of the time spent as juvenile at low (closed symbols) and high food (open symbols). The lines represent the regression of the linear model for each food treatment and the bars the± 95 % confidence intervals.

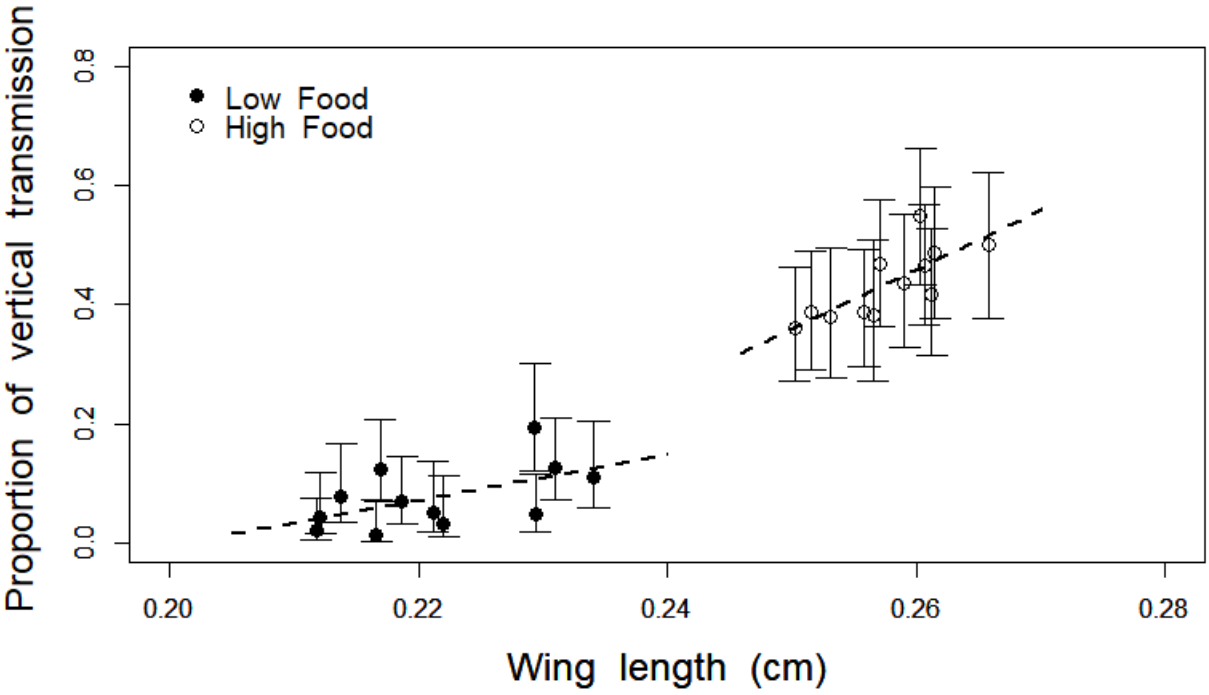

Figure 4 Relationship between vertical transmission and wing length (proxy for adult size). Dots represent the proportion of vertical transmission (proportion of individuals within the family enabling vertical transmission) as a function of the size of the wings at low (closed symbols) and high food (open symbols). The lines represent the regression of the linear model for the two food treatments and the bars the± 95 % confidence intervals.

3.4.4 Trade-off between vertical and horizontal transmission

The mean potential within families for the parasite to be transmitted vertically was negatively correlated with the average potential of horizontal transmission ($F=23.1$, $df=1$, $r^2=0.49$, $p<0.001$) (Figure 5). Although food treatment had no significant effect on the trade-off between vertical and horizontal transmission ($F=1.19$, $p=0.285$), this result is difficult to interpret because of the limited horizontal transmission at the high food level.

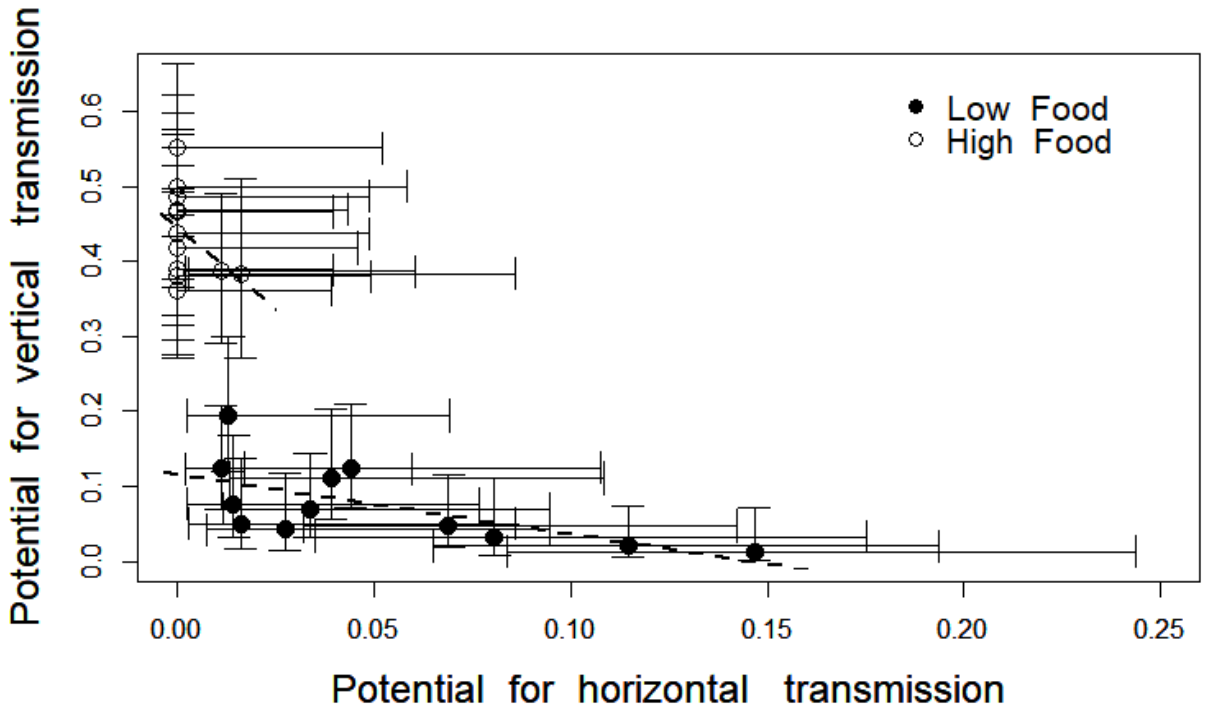

Figure 5 Potential for vertical transmission as a function of the potential for horizontal transmission in *Edhazardia aedis* at low food (closed symbols) and high food (open symbols). Each symbol represents the proportion of individuals within the family enabling vertical and horizontal transmission. The lines show the regression of the means, the bars represent the± 95 % confidence intervals.

3.4.5 Correlation between environments

The potential of a family for vertical transmission was uncorrelated between the two food treatments ($F_{1,10}= 0.0291$, $p=0.868$) (Figure 6). The analogous analysis for horizontal transmission would be meaningless, for almost all families allowed no horizontal transmission in the good-food environment.

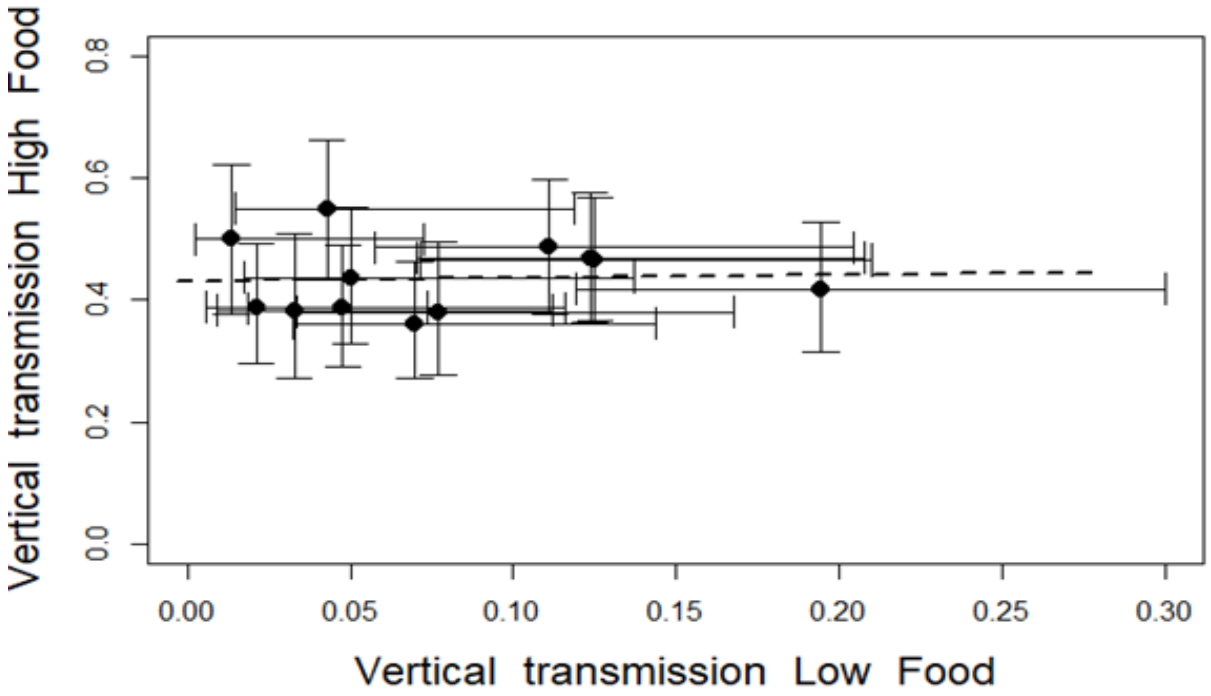

Figure 6 Correlation between the potential for vertical transmission (proportion of individuals within the family enabling vertical transmission) in high and low food conditions. Each dot represents the mean per family, the dashed line the regression line of the model and the bars the ± 95 % confidence intervals.

3.5 Discussion

Our results confirm that the transmission mode of *E. aedis* is affected by the growth conditions of the host. They also highlight the role of the host's genotype in linking its life-history to the parasite's transmission and in influencing the trade-offs underlying transmission mode.

Corroborating earlier studies (Koella et al. 1998; Agnew & Koella 1999), the parasite shows an adaptive response to changes of the host's development. Food conditions that slow growth increased the potential for horizontal transmission whereas favorable conditions (high food) increased vertical transmission. If growth is slow, adults are small and have low fecundity. Vertical transmission is therefore inefficient. However, poor food conditions also delay pupation, giving the parasite more time to complete its development from binucleate to uninucleate spores within a single larva. This enables the parasite to achieve a high load of horizontally transmitted spores, and thus to kill its host (Agnew & Koella 1999) and being transmitted efficiently. If growth is fast, in contrast, the parasite can expect that its host will lay many eggs; it therefore puts more emphasis on vertical transmission. Even if the rapid development does not allow the parasite to develop many spores, vertical transmission can be efficient, for only a few spores are necessary to infect all of the mosquito's eggs (Becnel et al. 1995). Transmission mode thus appears to be controlled by the production of uninucleate spores and the associated risk of larval mortality rather than by different allocation to the production of binucleate spores.

Note that our use of the phrase 'adaptive response' refers to (non-genetic) changes of the parasite's transmission mode. This does not imply that the parasite alters its development. Indeed, at least part of the adaptive response is simply a consequence of the timing of the parasite's fixed developmental pattern in the host's development, the parasite cannot produce a new generation of uninucleate spores without first going through a generation of binucleate spores. Uninucleate spores are therefore more likely to be found in more slowly developing larvae. An interesting implication of this idea is that the parasite has evolved its fixed developmental pattern in a way that optimises its transmission in its very variable environment (that is, host).

In addition to the response of the parasite to the host's environment, we found that the parasite changed its response according to the host's genetic background. The mosquito families differed with regard to the potential for transmission mode thus showing the presence of a genetic variation in the host for the transmission mode of the parasite. Genetic variation of a host for the transmission mode of a parasite has also been detected in the grass *Elymus hystrix* infected by the endophyte *Epichloë elymi* (Tintjer et al. 2008). In our case, the genetic basis for vertical transmission corroborates an experiment where artificial selection of the mosquito for more rapid or slower development affected the parasite's transmission mode (Koella & Agnew 1999).

Our data go further by suggesting that the parasite's transmission mode changes according to the genetic basis of several life-history traits such as larval survival, adult size and longevity. These genetic differences in life-history traits represent in themselves a first defence of the host against parasite threats, influencing the transmission mode which determines how parasites infect and exploit the hosts. The variation in the life-history traits of the host therefore has important consequences not only for the parasite but also for host's fitness. In particular, the families with the largest and longest-living individuals were most likely to enable vertical transmission (low virulence and higher fitness for the host), while the families with the slowest development were most likely to enable horizontal transmission (high virulence and lower fitness for the host). Although molecular and physiological effects were not measured, our results therefore suggest that several life-history traits are genetically linked to the complex infection-related and immunity genes. Such a trade-off between life-history traits and immune response is often assumed (Lee 2017) and supported by empirical studies (Soler et al. 2003; Kirschman et al. 2017), including in our host-parasite system (Desjardin et al. 2015). But the results also support the idea that the parasites switch in an adaptive way their transmission mode according not only to the environment, but also to the genotype of the host they are infecting. In families whose genetic background suggests high expected reproductive success, the parasite transmits vertically; in families with low reproductive success and long development the parasite switches to horizontal transmission. This switch according to genetic background is necessary to let the parasite to achieve high success, for the families that permit more vertical transmission permit less

horizontal transmission (at least in the low-food conditions, where it was possible to investigate this trade-off).

The trade-off between vertical and horizontal transmission has important consequences for the evolution of, both, the parasite and the host, in particular because horizontal transmission is associated with higher virulence than vertical transmission (Ebert & Herre 1996). For *E. aedis*, this association is necessary because of the parasite's life-cycle, and more generally this trade-off is predicted by evolutionary theory (Lipsitch et al. 1995; Lipsitch et al. 1996; Day& Proulx 2004; Ferdyn & Godelle 2005). First, there should be selection for hosts that enable more vertical and less horizontal transmission. This could lead in the long-term to the loss of horizontal transmission and therefore more effective vertical transmission. Indeed, this has been observed in an evolutionary experiment involving *P. caudatum* and *H. undulata* (Dusi et al. 2015). Second, since the host's genetic structure affects the ability of the parasite to transmit horizontally, it also affects the evolution of the parasite's virulence. In particular, with the expected selection of the host towards less damaging vertical transmission, we would expect a correlated response of the parasite to lower virulence thus reinforcing the evolution to vertical transmission. Third, while at high food hosts develop rapidly and almost always enable vertical transmission, we expect at low food and in the absence of parasite pressure that the host's life-history evolves to delayed age at maturity (Stearns & Koella 1986). Introducing parasites pressure impedes this evolution towards an optimal strategy by forcing the host towards vertical transmission with early maturity, although the associated low fecundity is not beneficial for either the host or the parasite. Under parasite pressure indeed, slow development hosts are more likely to die, whereas fast development host are favoured but with a smaller size than the optimum and consequently with a cost in fecundity.

We confirmed a response of *E. aedis* in its transmission strategy according to environmental conditions and the host's genotype. This response may be considered to be adaptive, for it leads to effective vertical transmission when the host is expected to achieve high fecundity and to more horizontal transmission when the host has a slow rate of development. Since mode of transmission is linked to the evolution of the parasite's virulence, the host's contribution to the trade-offs underlying transmission can influence considerably the epidemiology and evolution of parasites with mixed-mode transmission.

Chapter 4

Evolution under different host's availability of resources alters parasite's mode of transmission

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4.1 Abstract

Ecological conditions may greatly affect the relative importance of vertical and horizontal transmission, in particular for parasites with a mixed mode of transmission. High resource availability may favour fast host-growth conditions, while low resource availability may delay host's development. However, how these factors may favour the vertical or horizontal mode of transmission influencing parasite's evolution remains unclear. We test this idea using the microsporidia parasite Edhazardia aedis and its mosquito host Aedes aegypti. We let the parasite evolve for 10 generations in different host-growth conditions represented by 2 different constant availability of resources or changing the resources at each host's generation. The host was kept constant and not allowed to evolve. We finally test the lines of parasite obtained in two levels of food, representing the same or different conditions experienced during evolution. We measured the potential for vertical and horizontal transmission, the trade-off between the two, and the effect on host's life-history (juvenile development, adult size, and longevity). We found changes in the transmission of the parasite and in the life-history traits of the infected host; low resources during evolution increased vertical transmission, host's body size and longevity, fluctuating resources favoured horizontal transmission and delayed juvenile development, and high resources had no evident effects. These results illustrate how the transmission components of mixed-mode transmission parasite may quickly change and respond to different selective pressures imposed by the environmental conditions.

4.2 Introduction

Transmission mode is key to evolutionary parasitology and epidemiology, with important implications for host and parasite evolution. Despite so, there has been limited attention on how transmission mode evolves (Antonovics et al. 2017), with many studies focusing on co-evolution (Koella & Restif 2001; Boots et al. 2014) or the evolution of virulence (Alizon et al. 2009; Bull & Lauring 2014).

Two modes of transmission can be distinguished. Horizontal transmission is the spread of a parasites among hosts in a population, and it is expected to be related to high virulence when the exploitation of the host increases the rate of transmission of the parasite. In contrast, vertical transmission is the passage of the parasite from infected mother to offspring, and is usually associated with low virulence, for the parasite and the host align their evolutionary interests (Frank 1996). The conditions enabling vertical and horizontal transmission to coexist have been predicted by some theoretical models (Lipsitch et al. 1996; Ferdyn & Godelle 2005), which can be applied to mixed-mode transmission parasites (e.g. HIV, hepatitis B and C viruses).

In many parasites with a mixed mode of transmission, the investment in one transmission mode (either vertical or horizontal), will reduce the transmission with the other. For both transmission pathway cannot be simultaneously optimized, selection will operate on the component that maximizes the total transmission success. The two modes of transmission and the associated virulence may therefore experience different selective pressure. This trade-off, since the two modes of transmission are physiologically, developmentally or evolutionarily linked (Ebert & Mangin 1997, Messenger et al. 1999; Ebert 2013), has a fundamental role in a parasite's evolution and epidemiology. The reduction in host's fitness due to the nature of virulence of the horizontal component of the parasite, for example, may impose constraints on the shape of the trade-off and consequently influence the evolutionary outcomes (Lipsitch et al. 1995; Ferdy & Godelle 2005).

Ecological and demographic conditions greatly affect the relative importance of vertical and horizontal transmission, however how these factors favour one or the other transmission mode and influence the evolution of parasites remains unclear (Ebert 2013). Experimental evolution studies manipulated opportunities for the two transmission strategies of the parasites and obtained conflicting results. Rapidly growing populations of the host *Paramecium caudatum* (simulating high-growth conditions) selected for increased vertical transmission of the bacterial parasite *Holospora undulata* (Magalon et al. 2010). In addition, in these populations vertical and horizontal transmission were positively correlated compared to slow growing populations, suggesting that it was the quality rather than the quantity of parasite propagules to be selected. In the same study system, the bacterial parasite almost loss its horizontal component when evolved in condition promoting vertical transmission (Dusi et al.

2015). In contrast, changing the bacteria density of *Escherichia coli* for 500 generations gave no evidence for an increase in the horizontal transmission of a plasmid (Turner et al. 1998). The complex epidemiological and demographic process underlying the interactions between host and mixed-mode transmission parasites are likely reflected on the general lack of consistent pattern in the empirical data for the evolution of modes of transmission (Lipsitch et al. 1995; van den Bosch et al. 2010). Further, it is hard to generalize horizontal and vertical transmission strategies, for parasites species will responds differently to the same ecological settings in accordance to their developmental constraints.

The microsporidian parasite Edhazardia aedis, which can transmit horizontally among larvae of its host Aedes aegypti or vertically through the eggs of an infected female (Becnel et al. 1989), may help to better understand the evolution of transmission mode. The parasite has a developmentally fixed program and maintains vertical and horizontal transmission as an indispensable part of its life cycle. Indeed, during the within-host development, it alternates the production of specific spores that are strictly associated to vertical or horizontal transmission. In good growth conditions, larvae develop quickly and emerge as adults at a time where the parasite have reached a high number of vertically transmitted spores. In bad growth conditions, larval development is delayed, and the parasite has more time to further produce the horizontally transmitted spores that kill the mosquito during its juvenile stage before emergence (Koella et al. 1998; Agnew & Koella 1999). The life cycle of E. aedis is dominated by the trade-off between the two transmission mode; horizontal transmission requires the death of the larvae, which in turn precludes the vertical transmission. Occasionally, following horizontal infection, the production of vertically transmitted spores goes on to produce new horizontally transmitted ones before the emergence of the adult. If the juvenile is killed, this can lead again to horizontal transmission without the requirement of vertical transmission (Becnel et al. 1989). The potential to complete the life cycle within a single larva suggests that the development of E. aedis is not explicitly linked to the one of Ae. aegypti (Agnew & Koella 1999). Although the host has some control over parasite's life cycle, E. aedis is still controlling its mode of transmission in some measures (Koella & Agnew 1999). This raises the question whether selection can still operate on the efficacy of its transmission mode.

We let the parasite evolve under different host-growth conditions represented by different resource availability. Different levels of resource determine how fast the host develops, and thus represented restriction opportunities for either the horizontal or vertical transmission of the parasite. Low food conditions slow larval growth, whereas high food conditions enhance a fast development into adults. At low food, juvenile mortality is high and vertical transmission is difficult. Thus, there should be selection to increase the size and have more vertical transmission in the mosquitoes able to emerge and leave the harsh aquatic environment. In favourable host-growth conditions (high food), there

should be selection for fast-growing parasites in the vertically infected offspring. These parasites will be able to kill the host faster increasing their chances for a successful horizontal transmission and to spread in the juvenile population. The parasite was not a priori forced into one particular mode of transmission, but it followed its natural life-cycle with the alternation of horizontal and vertical infective spores. The host was kept "constant" and not allowed to evolve. In addition, we asked whether rapid fluctuation of the transmission opportunities (i.e. alternation of different resource availability for the host at each generation) enhance one of the two modes of transmission. Finally, we measured the condition of the parasite after 10 generations of evolution. We tested its potential for vertical and horizontal transmission, the trade-off between the two, and the effect on host's life-history (juvenile development, adult size, and longevity).

4.3 Materials and methods

The whole experiment (experimental evolution and the final test) was run in a climate chamber set at 26 °C, 70% humidity, 12 h:12 h light:dark photoperiod.

4.3.1 Experimental system

For our experiment we used the yellow fever mosquito *Aedes aegypti* (obtained from Patrick Guérin, University of Neuchâtel) and its specific microsporidia parasite *Edhazardia aedis* (provided by J. J. Becnel, USDA, Gainesville, USA). The genetics, physiology, and ecology of *Ae. aegypti* are well known (Christophers 1960; Nene et al. 2007) due to its impact on human health. This mosquito species is globally distributed in all tropical and subtropical regions and is the main vector of many arbovirus infecting humans (Petersen et al. 2016). The larvae grow in natural or artificial containers and during their development are often challenged by periods of nutrient restriction and competition (Chan et al. 1971; Reiskind & Lounibos 2009). The eggs present a desiccation stage and can be stored for some months at standard lab conditions (Munstermann 1997).

E. aedis has a complex life cycle with a mixed-mode transmission (Becnel et al. 1989; Becnel 1993 & Johnson; Becnel et al. 1995; Johnson et al. 1997; Desjardins et al. 2015). It produces different types of spores which are functionally and morphologically distinguishable in uninucleate spores for horizontal transmission and binucleate spores for vertical transmission. Mosquito larvae ingest uninucleate spores of *E. aedis* with their food, resulting in the infection of the gut and epithelial cells. The infection subsequently spreads to other tissues, and eventually the parasite begins to produce binucleate spores in the oenocyte cells. These spores inoculating the oocytes of a female are the responsible for vertical transmission (transovarial transmission). Thus, the males represent a dead-end route for *E. aedis*. Almost the totality of the eggs laid by an infected female (>90%) will result in larvae carrying the parasite (Bencel et al. 1995). Once the eggs hatch, a new generation of uninucleate spores is produced

in the growing larvae with few individuals surviving beyond pupation. Horizontal transmission occurs when the larvae ingest the uninucleate spores released in the aquatic environment after the infected larva dies, the spores can remain infective for about 48 hours (Nasci et al. 1992). This form of transmission is enhanced by food deprivation or high parasite load (Agnew & Koella 1998). A repeated horizontal transmission is possible when the parasite produces binucleate spores in the larva followed by uninucleate spores which finally kill the host.

4.3.2 Experimental design

Experimental Evolution

We let *E. aedis* evolve for 10 generations in three different environments, constant favourable host growth conditions (high food availability), constant detrimental host growth conditions (low food availability) and an alternation of these two conditions which were changed at each host's generation. Each of these three treatments were replicated five times for a total of 15 lines of parasite.

The first generation of the experimental evolution was obtained by synchronously hatching colony eggs of *A. aegypti* under condition of low air pressure and haphazardly assigning 200 larvae to each line. The remaining larvae were used to start a new standard uninfected colony, which run in parallel to the infected lines and followed the same generation timing. Further, this colony supplied the larvae that were infected at each generation as it was only the parasite to evolve, whereas the host was kept "constant" and not allowed to coevolve with the parasite.

The larvae were split in groups of 50 individuals and reared for the first four days in 4 petri dishes (8 cm diameter) containing 30 mL of deionized water. They were fed daily with either our standardized ration of TetraMin baby™ fish food (age 0 (day of hatching): 0.06 mg/larva, age 1: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg, from age 5 onwards: 0.32 mg), or with the half of the standard diet. These food regimes represented the high and low availability of resources for the host. Seventy-two hours after hatching, we exposed the larvae of each petri dish to infection adding 2×10^5 of uninucleate spores in 1 mL. This quantity of spores assures a successful infection and prevent a high juvenile mortality rate. The spores had been harvested from vertically infected larvae hatched 7 days earlier than the day of infection and originated from the same clutch of infected eggs. Thus, we considered that all the lines of the three evolutionary treatments were infected with the same parasite (same pool of spores). We crushed and homogenized fifteen of these larvae in an Eppendorf tube with 1mL of deionized water to determine the number of uninucleate spores using a hemocytometer and a phase-contrast microscope (Zeiss Axio Lab.A1). Twenty-four hours after infection, four groups of 50 larvae were transferred into 200 x 150 x 50 mm plastic tray containing 1 L of deionized water, each tray represented a different line of parasite.

The pupae were moved to 30 x 30 x 30 cm size cages containing a 10% sugar solution and an oviposition cup with a filter paper. No studies exist on the potential effect of males on the fecundity and longevity of the females when the entire population is infected with *E. aedis*. Thus, emergence was checked three times a day and the males were removed with an aspirator from the cages before they had the chance to copulate. These were replaced with 25 males originating from the standard uninfected colony that were run at the same time as the infected lines. The mosquitoes were blood fed for 10 minutes on GZ's arms five and seven days after the day when the 75% of the individuals of a given line pupated. The filter papers with the eggs were removed every 2 days and stored in a petri dish in the climate chamber until the start of a new generation. Starting from the second generation, the eggs of each line were hatched under low air pressure and, 7 days after, 15 individuals were used to obtain the infection solution to infect the three-days old larvae supplied by the standard uninfected colony.

Measuring final conditions

We tested each of the line of *E. aedis* obtained after 10 generations of evolution using individually reared mosquitoes in two food levels (high and low food), the favourable and detrimental host growth conditions. Using this approach (except for the lines that experienced an alternation of the diet at each generation) the parasite experienced once more the conditions in which it evolved for 10 generations or the alternative treatment. We measured the transmission potential of the lines by counting the presence of uninucleate and binucleate spores in the dead individuals and the effect of the parasite on the life-history traits of the host. In the last generation, all the females from one of the five parasite lines experiencing constant detrimental host growth conditions died before to lay any eggs. Thus, that line was lost, and we finally had 14 lines of parasite. Eggs from the colony were simultaneously hatched with a desiccator under low pressure condition. A total of 3804 larvae were haphazardly moved to and individually reared in 12-well tissue-culture plates filled with 3 mL of deionized water. Half of the larvae were assigned to a high food regime, and the remaining half to a low food regime, each plate contained larvae from both treatments. The larvae assigned to the high food were fed daily with the 100% of our standardized ration of TetraMin baby™ fish food (age 0 (day of hatching): 0.06 mg/larva, age 1: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg, from age 5 onwards: 0.32 mg), and the low food larvae received the 50% of the standardized ration.

Forty-eight hours after hatching, each larva received 400 uninucleate spores in 100 µL of one of the fourteen lines of parasite (average of 135 individuals per treatment per line). The spores had been obtained by hatching the vertically infected larvae of the lines 7 days earlier than the day of infection. We crushed and homogenized fifteen of these larvae in an Eppendorf tube adding 1mL of deionized water, and the concentration of the uninucleate spores was determined using a hemocytometer and

a phase-contrast microscope (Zeiss Axio Lab.A1). We moved the pupae individually into Falcon tubes and we provided the emerging adults with a cotton wool soaked with 10% sugar solution which we replaced every 6 days. The survival of all the individuals (larvae, pupae and adults) was checked daily. We collected the dead mosquitoes into 2 mL Eppendorf tubes and stored them at -20°C until further investigation. We stopped the experiment at day 23 after hatching and all the individuals alive at that moment were sacrificed and moved in a freezer at -20°C. The spore load was measured for each individual adding 0.1 mL of deionized water and homogenizing the samples with a TissueLyser LT-QIAGEN. We counted the spores sampling 4 µL of the obtained solution and using a hemocytometer placed under a phase contrast microscope (Zeiss Axio Lab.A1).

The treatment status of the samples was unknown during the counting. We measured juvenile development (days spent in the aquatic environment as larvae and/or pupae), longevity and wing length as proxy for adult body size (Koella & Lyimo 1996). The wings were removed and mounted on microscope slides, thereafter they were digitally scanned and measured with the software IMAGEJ. Males were removed from all analysis leaving us with 3283 mosquitoes.

4.3.3 Statistical analysis

We tested whether the proportion of females and juveniles was affected by the food level, the evolutionary treatment and their interaction using a generalized linear mixed effect model with binomial distribution. These explanatory variables were considered as fixed factors, we added the parasite lines (replicates) as random factor. Mosquitoes enabled horizontal transmission if they died during their juvenile stage harbouring uninucleate spores, and that they did not enable horizontal transmission when they carried no uninucleate spores or they emerged as adults. Adult females carrying binucleate spores enabled vertical transmission, and they did not enable vertical transmission otherwise. We analysed the horizontal and vertical transmission strategy of the parasite separately. We used a generalized linear mixed effect model with binomial distribution that included food level, the treatment during the evolutionary history of the parasite (resource availability of the host) and their interaction as fixed factor. The parasite lines were considered as random factor. We investigated a possible trade-off between vertical and horizontal transmission with a linear regression clustering the lines and using the means of each evolutionary treatment.

The effect of the parasite on juvenile development (days spent in the aquatic environment as larvae and/or pupae) and adult size (wing length) was analysed with a linear mixed effect model that included food, evolutionary treatment and their interaction as fixed factors, and parasite lines as random factor. We analysed longevity with mixed effect survival analysis (cox-proportional hazard) considering food,

evolutionary treatment and their interaction as fixed factors, parasite lines were included as random effect. A significant interaction between evolutionary treatment and food level indicated differences due to the evolution of the parasite.

All the statistical analysis was performed with the R version 3.4.2. We used, the lme4 (Bates et al. 2014) and car (Fox & Weisberg 2011) packages for the mixed effect models, and the coxme package for the survival analysis (Therneau 2018).

4.4 Results

4.4.1 Proportion of juveniles/females

A total of 2847 out of 3283 (75 %) individuals died as juveniles (Figure 2), and 436 out of 3283 (15 %) females emerged in the final test (Figure 3). The food level strongly affected juvenile mortality, with 22 % (c.i. 20-24) of the well-fed larvae and 5 % (c.i. 4-6) of the larvae fed on low food surviving to emergence (χ^2=176.32, df=1, p<0.001), but not the evolutionary treatment (HF: 12.1%, 10-14; LF: 15% 13-17; Alt: 12.8%, 10-15; χ^2=1.50, df=2, p=0.47) (Figure 1 and 2). The evolutionary conditions interacted to determine juvenile mortality, though the effect was not quite significant (χ^2=5.31, df=2, p=0.07), with the highest proportion of individuals emerging in the high food conditions when infected with parasite's lines experiencing low level of food during evolution (26 %, 22-30) and the lowest proportion in the low food level with the high food evolutionary lines of parasite (3.9%, 2-5)

4.4.2 Vertical and horizontal transmission

The horizontal transmission increased of a fivefold factor from the high food conditions (2.8 %, 2-3.8) to the low level of food (14 %, 13-16) (χ^2=112.02, df=1, p<0.001), but it was not affected by the evolutionary treatment (HF: 8%, 6.7-9.7; LF: 8.8%, 7.1-10.7; Alt: 10.7%, 9-12.8; χ^2=1.19, df=2, p=0.55) or by its interaction with food (χ^2=0.72, df=2, p=0.69) (Figure 1).

The evolutionary treatment was not influencing the vertical transmission of *E. aedis* (HF: 8.9%, 7.5-10.6; LF: 10.6%, .,8-12.7; Alt: 9.1%, 7.5-11.1; χ^2=0.65, df=2, p=0.72), in contrast the high food treatment highly increased the probability of harbouring binucleates spores of a more than a fivefold factor (high food: 16.9%, 15.1-18.9; low food: 3%, 2.3-3.9; χ^2=145.6, df=1, p<0.001) (Figure 2). The interaction between the food and the evolutionary treatment (so whether the effect of food on the success of the vertical transmission depended on the evolutionary regime of the parasite) was significant (χ^2=6.22, df=2, p=0.04), with highest and lowest potential for vertical transmission reached by the lines of parasite constantly subjected to food restriction during evolution and tested at respectively low (20%, 16.8-24) and high food (2.4%, 1.4-4.1). The mean potential within evolutionary treatments for the parasite to be transmitted vertically was negatively correlated with the average horizontal

transmission (F=26.31, df=1, r^2=0.83, p=0.006) (i.e. trade-off between vertical and horizontal transmission).

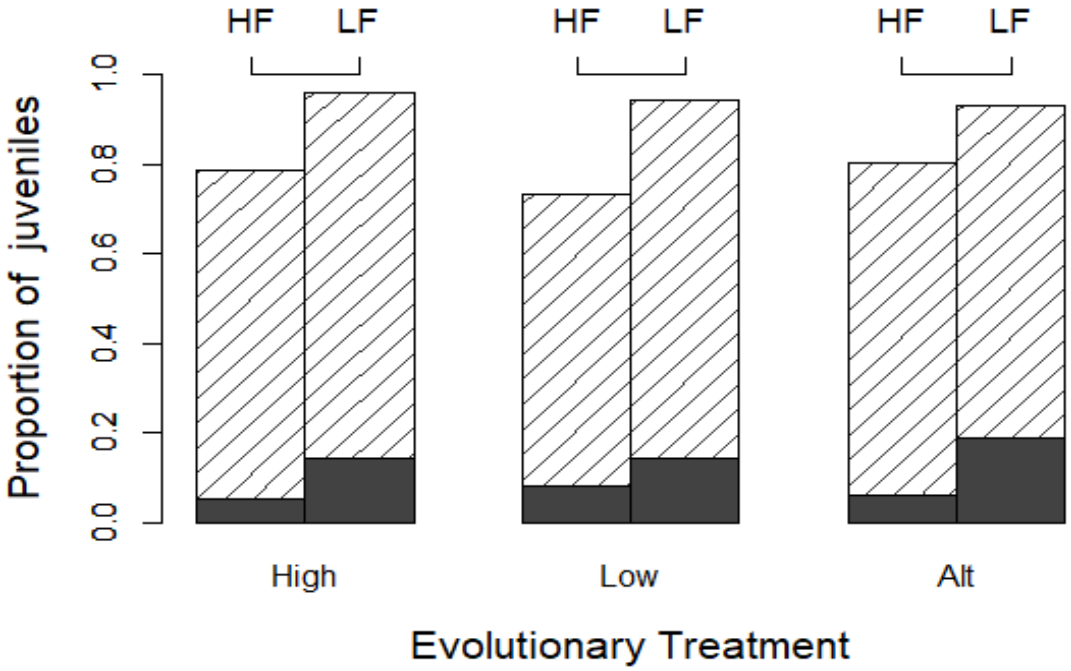

Figure 1 The proportion of individuals of all lines pooled by evolutionary treatment dying as juveniles (dashed bars), and of those the proportion harbouring spores and affording the potential for horizontal transmission for *E.aedis* (dark grey). The evolutionaty treatment represent the growth condition of the host during the evolution of the parasite, high availability of resources (High), low availability of resources (Low), and alternation of high and low availability at each generation (Alt). HF and LF represents respectively the high and low food regime in the final test.

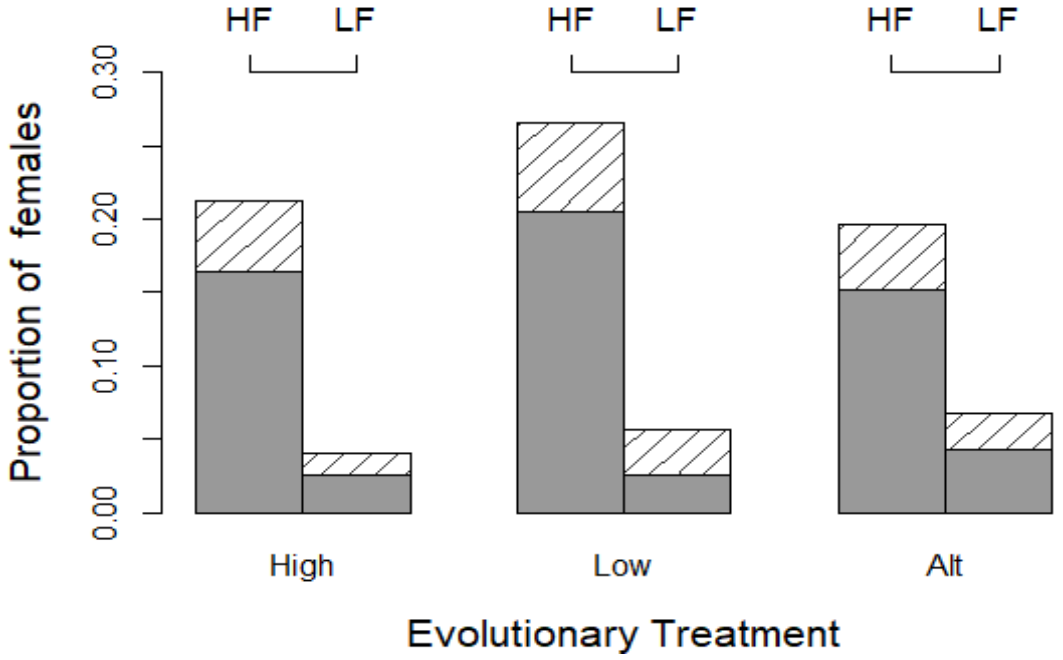

Figure 2 The proportion of females emerging of all lines pooled by evolutionary treatment (dashed bars), and of those the proportion harbouring spores and affording the potential for vertical transmission for *E.aedis* (light grey). The evolutionaty treatment represent the growth condition of the host during the evolution of the parasite, high availability of resources (High), low availability of resources (Low), and alternation of high and low availability at each generation (Alt). HF and LF represents respectively the high and low food regime in the final test.

4.4.3 Host's life-history traits

Juvenile development

Juvenile development was delayed of 2 and a half days in the low level of food compared to the high food (low food: 10 days, 9.9-10.1; high food: 7.47 days, 7.4-7.5; χ^2=180.4, df=1, p<0.001). The interaction between food and evolutionary treatment was significant (χ^2=8.16, df=2, p=0.016) with the mosquitoes in low food conditions infected by the parasite exposed to the fluctuation in resource availability during evolution delaying their development more than the other evolutionary treatment in high (7.57 days, 7.4-7.7) and low food (10.4 days, 10.2, 10.6) as shown in Figure 3A. The evolutionary treatment alone was not affecting the juvenile development (HF: 8.7 days, 8.6-8.8; LF: 8.7 days, 8.6-8.8; Alt: 9 days, 8.9-9.2; χ^2=1.88, df=2, p=0.39).

Adult size

The adult females from the high level of food were larger (2.84 mm, 2.84-2.85) than the ones from the low level of food (2.56 mm, 2.56-2.57) (χ^2=243.04, df=1, p<0.001), but this depended also on the evolutionary conditions experienced by the parasite (interaction food*evolution: χ^2=9.30, df=2, p=0.009). The females infected with the parasite experiencing low resources during evolution had the largest size at high food level (2.86 mm, 2.84-2.87) and the smallest size at low food level (2.51 mm, 2.49-2.53) (Figure 3B). There was a strong tendency for the evolutionary treatment to affect adult size (χ^2=5.78, df=2, p=0.055) with mosquitoes infected by the parasite evolved in constant high resources line reaching an average of 2.81 mm (c.i. 2.80-2.82), in constant low resources 2.79 mm (2.77-2.80), and in the alternation of resources 2.76 mm (2.75-2.77).

Longevity

The 87 % of the mosquitoes in which the infections were confirmed died within 14 days after hatching, with only 11 individuals (0.6%) surviving up to the end of the experiment (day 23 after hatching). The longevity of the host was highly affected by the food level (χ^2=11.52, df=1, p<0.001), 15 % of the individuals reared in high food were still alive at day 15, whereas only the 10 % survived in low food (Figure 4) The evolutionary lines of the parasite significantly affected mortality (χ^2=6.01, df=2, p=0.04) with an average of life span of 10.9 days (10.7-11.1) for the high resource lines, 11.54 days (11.2-11.8) for the low resource lines ,and 11.57 days (11.2-11.8) for the alternation of resource lines. The food level and the evolutionary treatment interacted (χ^2=11.72, df=2, p=0.002) with mosquitoes infected with the lines evolved under alternated availability resources and tested at low food living in average the longest (12 days, 11.7-12.4), and the lines evolved with high availability of resources and tested at high food living the shortest (10.56 days, 10.2-10.8).

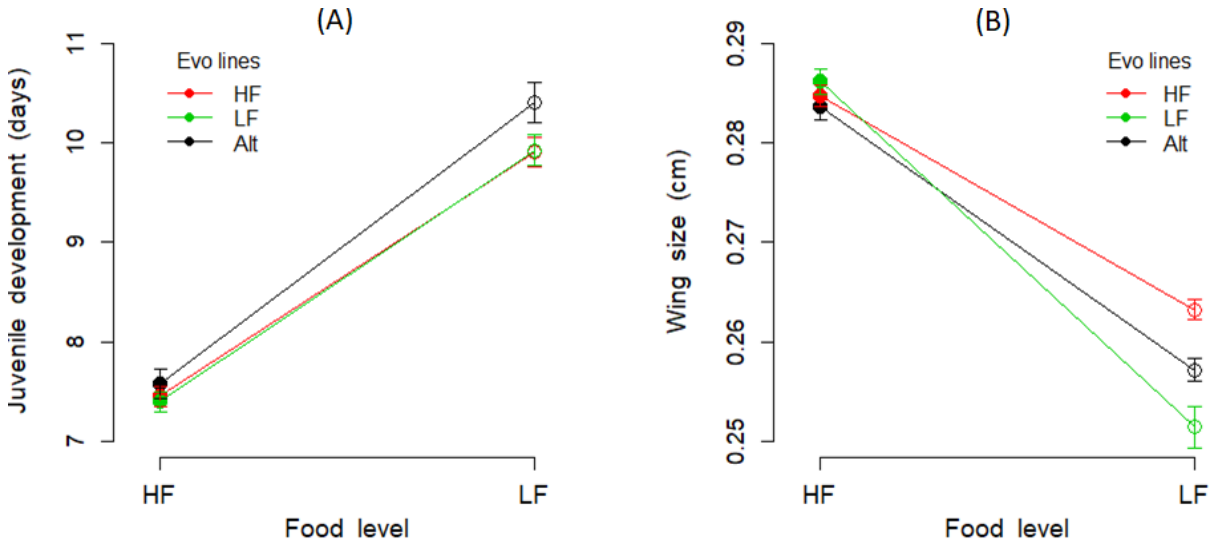

Figure 3 Interaction plot showing the effect of the parasite after 10 generations of evolution in different environmental conditions on the life-history traits of the host. Mosquitoes infected with different lines of the same evolutionary treatment of parasite are pooled, with each point representing the mean of the host's trait ± standard error. In (A) the mean days spent in the water and (B) the mean size of the females. The different evolutionary treatment is shown in different colours, in red the individuals exposed to the parasite evolved in high resource availability, in green low resource availability, and in black the alternation of resources at each generation. Full and empty dots are the food level, respectively high (HF) and low (LF) food, in the final test.

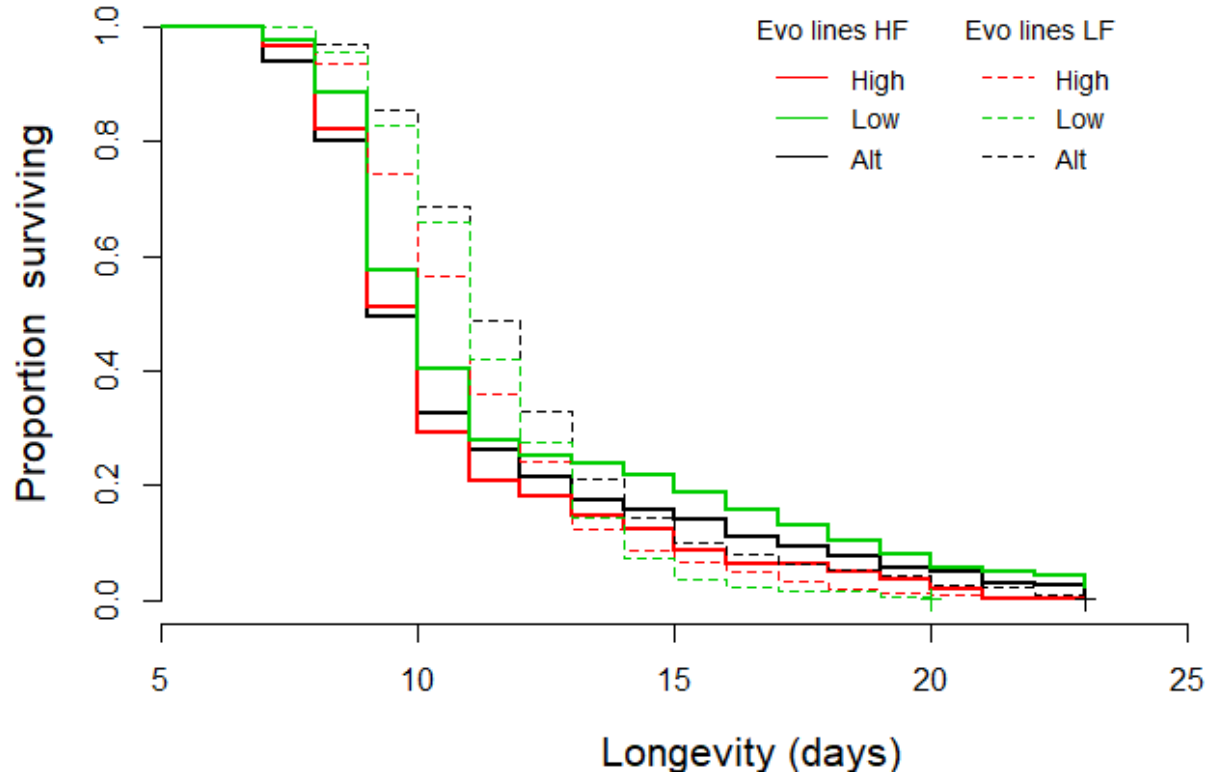

Figure 4 Survival curves of the mosquitoes exposed to lines of parasite evolved in different ecological settings, high resource availability (red), low resource availability (green) and alternation of this two resources (black). Mosquitoes infected with different lines of the same evolutionary treatment of the parasite are pooled. Full and dashed lines represent the food level, respectively high (HF) and low (LF) food, in the final test.

4.5 Discussion

Our results show a change in the transmission of *E. aedis* after 10 generations of evolution in different host-growth conditions (i.e. resource availability) and in the life-history traits of the infected host. To our knowledge this is the first example of such an evolutionary response in a eukaryote parasite (unicellular fungus), as previous studies considered the evolution of transmission mode on viruses and bacteria (Stewart et al. 2005; Magalon et al. 2010; Pagan et al. 2014; Dusi et al. 2015; Berngruber et al. 2015). We discuss the findings of our evolutionary experiment focusing on the vertical and horizontal transmission.

The parasite *E. aedis* increased the potential for vertical transmission when evolved under low availability of resources, few infected females were able to emergence, take a blood meal and lay eggs (data not shown). These were the only mosquitoes capable of producing binucleate spores via infected eggs in that generation. The increase vertical transmission was particularly evident when such evolutionary treatment was tested at high food level, with more females emerging and harbouring binucleate spores (Agnew & Koella 1999). The mosquitoes infected with these lines of parasite which always experienced food restriction during evolution, were the largest in high food level and the smallest in food restriction (Figure 3B). Thus, the parasite evolved and maximized its vertical transmission in two ways. Firstly, it directly enhanced the production of binucleate spores, particularly in good host-growth conditions. In the mosquitoes infected with these strains it was indeed more likely to find binucleate spores. Secondly, it affected the body size of the host, which may influence the transmission mode that determines how parasites infect and exploit the hosts. In *Ae. aegypti*, body size is positively correlated with long-living individuals, suggesting high expected reproductive success (Briegel 1990; Naksathit & Scott 1998). Confirming the idea that they were the most likely to enable vertical transmission, these individuals lived longer than the ones exposed to the evolutionary lines of parasite evolved under constant high food and had similar longevity to the ones infected with the parasite experiencing variable conditions (Figure 4). In addition, when the evolutionary lines of *E. aedis* experiencing low host's resources were tested at low food, the parasite was optimizing the vertical component by having the same proportion of females harbouring binucleate spores than the other lines, despite the smallest body size (Figure 3A) and the same juvenile development of the high-resources evolutionary lines (Figure 1).

The high availability of resources during evolution did not favour or changed the horizontal component of the parasite. Horizontal transmission was predominant when the parasite lines were tested at low food level in the final experiment, with the hosts delaying their juvenile development and increasing the probability of carrying uninucleate spores as expected (Agnew & Koella 1999), but this was the

same for all the evolutionary treatment. However, the overall higher proportion of juveniles carrying uninucleate spores and the delayed juvenile development (particularly evident at low level of food, Figure 3A), may suggest that the fluctuation of resources during evolution was selecting for an increase in horizontal transmission. In fact, it has been shown that delayed pupation is associated with parasite's potential for horizontal transmission (Agnew & Koella 1999; Koella & Agnew 1999). In an unpredictable environment similar to the natural conditions of the system (Christophers 1960; Reiskind & Lounibos 2009), with food restriction and limited resources available due to juvenile competition, the parasite may prefer to invest more in horizontal transmission. In this way, it may spread more efficiently among the juveniles, infect new individuals and persist in the host's population. Once the ecological conditions, and consequently the host's conditions improve, the larvae will grow and emerge faster, and the parasite will be still able to assure some vertical transmission.

A possible explanation for the absence of a response in the parasite evolved with constant high resources is that we simultaneously applied different selective pressures. For almost all of the females of this treatment emerged and laid eggs (vertical transmission) at each generation (data not show) differently from the treatment with the alternation of resources, it is possible that effect of the fast-growing parasites in the vertical infected larvae (horizontal transmission) was diluted. As final result, in this treatment the parasite transmission mode and the overall transmission success remained stable over evolution, for the different divergent selective pressure acting on the vertical and horizontal transmission prevented any significative change.

The restriction opportunities were affecting the evolution of parasite transmission mode with direct and undirect evidence for respectively vertical and horizontal transmission. The evolution of parasite transmission mode remains a challenging question. Our results illustrate the complexity to predict, and empirically test the hypotheses (Antonovics 2017), particularly for mixed-mode transmission parasites (Ebert 2013). The parasite *E. aedis* responded to selection and affected the juvenile development, size, and longevity (reproductive success) of *Ae. aegypti*. This highlight the presence of genetic variation for transmission mode in a eukaryote parasite with a developmental fixed program. Finally, this show how the different transmission component of a parasite with mixed mode of transmission may quickly change and respond to environmental restriction opportunities such as host's resource availability in few generations, with important consequences for the evolution of host-parasite interactions.

Chapter 5

The effect of parasite infection on the recombination rate of the yellow fever mosquito *Aedes aegypti*

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5.1 Abstract

Sex and recombination generate new genetic combinations and may help an individual infected by a parasite to protect its offspring from being infected. While this is often used to understand the evolutionary forces underlying the maintenance of sex and recombination, it also suggests that infected individuals should increase plastically their recombination rate. We tested the latter idea with the mosquito Aedes aegypti and asked whether females infected by the microsporidian Vavraia culicis were more likely to have recombinant offspring than uninfected females. To measure the rate of recombination over a chromosome we analysed combinations of microsatellites in infected and uninfected females, in the males they copulated with and in their offspring. As predicted, the infected females were more likely to have recombinant offspring than the uninfected ones. These results show the ability of a female to diversify her offspring in response to parasitic infection by plastically increasing her recombination rate.

5.2 Introduction

Genetic recombination shuffles the genes of adults to generate novel genotypes in offspring. This has the disadvantage of breaking up the combinations of genes that were beneficial to the adults (Maynard Smith 1978; Agrawal 2006) but may have the advantage that recombination can help to protect an individual's offspring against parasites (Hamilton et al. 1990; Otto & Nuismer 2004; Lively 2010). It is, for example, often the case that a given parasite can infect only a few genotypes of its host species (Lambrechts et al. 2006). Therefore, a parasite that has infected a host will be less able to infect that individual's offspring, if genetic recombination has led to sufficiently large differences between the infected individual and its offspring.

This forms the basis of several ideas about the maintenance of sexual reproduction. Since hosts gain an advantage by producing offspring with rare and unusual genetic combinations, which are protected to some degree from being infected by their parasites, selection will tend to maintain a high level of recombination in populations where parasites are common (Koskella & Lively 2007; Koskella & Lively 2009). This is supported, for example, by experiments with the flour beetle *Tribolium castaneum* and the microsporidian *Nosema whitei* (Fischer & Schmid-Hempel 2005; Greef & Schmid-Hempel 2010; Kerstes et al. 2012). The flour beetle reproduces strictly sexually, but its rate of recombination can vary. After experimental populations of the beetle had been allowed to evolve for 11 generations either in parasite-free habitats or under attack by the co-evolving microsporidian, the frequency of recombination was greater in the lines that had coevolved with *Nosema whitei* than in the parasite-free ones

Alternatively, rather than waiting for evolutionary pressure to change the rate of recombination, we might expect that infected individuals plastically increase their recombination to protect their offspring. Indeed, organisms change their recombination rate according to the environment. It was, for example, observed more than 100 years ago (Bridges 1915) that the rate of recombination of Drosophila melanogaster differs according to temperature, humidity, diet, age, and social status (reviewed in Stevison et al. 2017). Such plasticity in the rate of recombination is also found in other animals such as nematodes or grasshoppers, in plants such as tomatoes and tobacco, and in humans (Fabre & Roman 1977; Abdullah & Borts 2001; Lim et al. 2008; Maguire 1968; Lu & Chiu 1976; Church & Wimber 1969; Zuchenko et al. 1986; Balyaev 1982; Lenzi et al. 2015; Campbell et al. 2015). However, the phenotypic plasticity of recombination rate in response to parasites is rarely considered. While the few studies in plants (Lucht et al. 2002; Kovalchuk 2003; Andronic 2012) all found that individuals infected by viruses or with an oomycete have a higher rate of recombination than uninfected ones, the two existing studies in animals gave conflicting results (Singh et al. 2015; Dumont et al. 2015). In response to either bacteria or parasitic wasps, *Drosophila melanogaster* increase the recombinant

fraction of its descendants by transmission distortion of the recombinant chromatids (Singh et al. 2015), whereas recombination in house mice is not affected by bacterial infection (Dumont et al. 2015).

The aim of our study was to provide further evidence for a plastic increase of the rate of recombination in response to parasitic infection. We therefore asked how the recombination rate of the mosquito *Aedes aegypti* changes if it is infected by the microsporidian *Vavraia culicis*.

5.3 Material and Methods

5.3.1 Experimental system

We used the UGAL strain of the mosquito *Aedes aegypti*, which was provided by P. Guérin (University of Neuchâtel), and its microsporidian parasite *Vavraia culicis*, which was provided by J. J. Becnel (USDA, Gainesville, USA). *Aedes aegypti* is the main vector of yellow fever, dengue, chikungunya and Zika viruses (Petersen et al. 2016). Due to the impact on human health its physiology, genetics, and ecology are well known (Christophers 1960; Nene et al. 2007). It is ubiquitous in the tropics and subtropics, where the larvae growing in natural or artificial containers encounter periods of nutrient restriction and competition (Chan et al. 1971; Reiskind & Lounibos 2009). The eggs resist desiccation and can be stored for several months. After a single mating the females lay eggs throughout their lifespan (Munstermann 1997, Oliva et al.2014). *Vavraia culicis* is an obligate endocellular parasite of several genera of mosquitoes (Weiser & Coluzzi 1972). Infection occurs in the aquatic environment when the mosquito larvae ingest the spores with their food. The parasite penetrates the gut and epithelial cells, undergoes a series of developmental stages and finally produces the infectious spores. These are usually transmitted horizontally when larvae or pupae die in the water. If infected mosquitoes survive to adulthood, the spores can adhere to the surface of the eggs and infect newly hatched larvae (Andreadis 2007) or the mosquitoes dying on the surface of a larval site can release the spores.

5.3.2 Experimental design

The purpose of this study was to compare the recombination rate of infected and uninfected females. To measure the rate of recombination, we genotyped microsatellites of mothers, fathers and their offspring, and measured for each pair of microsatellites the proportion of offspring that were recombinant (Figure 1). Throughout the experiment, the mosquitoes were maintained at 26 °C, 70% humidity, 12 h:12 h light:dark photoperiod.

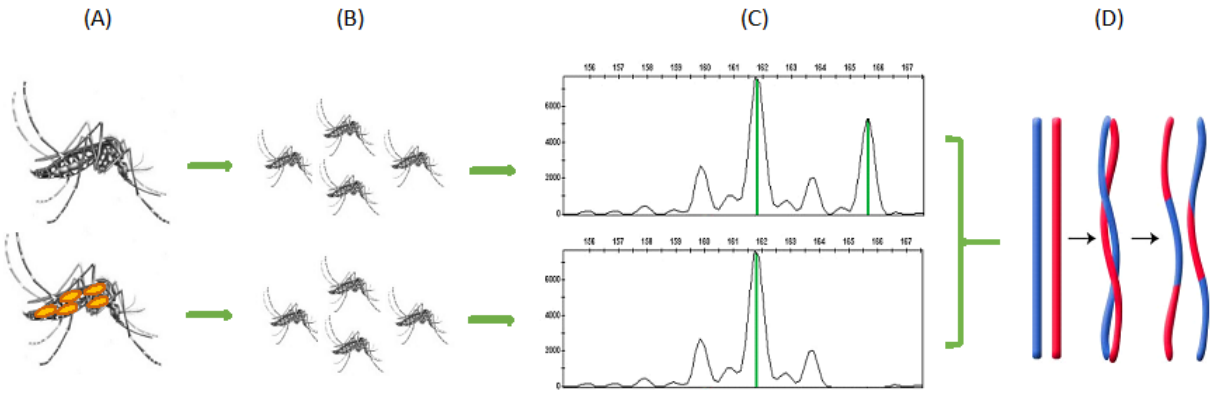

Figure 1 Schematic path of the experimental design. (A) Females of *Aedes aegypti* were reared, and half of them were infected with the microsporidian parasite *Vavraia culicis* (represented by the dots). (B) For each parent and offspring, we conducted DNA extraction and PCR on 4 or 6 microsatellites loci of the chromosome three. (C) The samples were run through capillary electrophoresis for allele detection. The genetic profiles, i.e. determination of homozygosity or heterozygosity at each locus, were obtained using the Genemapper® software. (D) Recombination rate of the mothers was measured with the CRIMAP software.

The experiment was performed in 2 blocks with the same experimental procedure except for the number of microsatellites analysed (Table 1). In the first block we used four microsatellites (A, B, D and F); in the second we used two additional ones (C and E) (for further information see Lovin et al. 2009: Additional file 1). We chose microsatellites that are located on the same chromosome (chromosome three), for the markers on different chromosomes can be expected to recombine freely, leading to a recombination fraction that is not affected by the environment. The forward primers of each microsatellite were modified with a commercial fluorochrome (Table 1) for genotyping by capillary electrophoresis.

Table 1 Primers characteristics of the 6 microsatellites markers used (modified by Lovin et al, 2009). We called the microsatellites called A to F for simplification (column ID).

ID	GenBank accession #	Map Location	Locus	Size (bp)	Flurochrome	Forward primer 5'-3'	Reverse primer 5'-3'
A	T58329	3-00-0	301CT1	207	AT 532	CTGAACGCGCCATAAATTCT	AGGAGTTCGTCCCAAGACAA
B	BM005489	3-23.5	766ATT1	301	FAM (Fluorescein)	TGCAAAGTCGAAGCAACAAG	GAATGCCATTTGCCTTCA
C	R47184	3-32.1	69TGA1	214	FAM (Fluorescein)	CACCTCCGCTAGAGAACTGG	CGAATAGGGCAATCCTGAAA
D	DV309356	3-43.7	86AC1	257	AT 532	GCGAATCGGTTCCCATAGTA	ACCCATCGAATTTCCATTCA
E	AF324863	3-50.0	217CTT1	257	FAM (Fluorescein)	TGGACTTCCCCAGATGCAATGA	CAACACGGAAGCAAAGTTGA
F	L12389	3-57.1	201AAT1	336	AT 550	GATCGTTCGACAGCATCTGA	GGAAAGCTCATCGCCTACTG

Parental generation

Eggs of *Ae. aegypti* from the colony were synchronously hatched under low pressure conditions, and 400 haphazardly chosen larvae were individually reared in the wells of 12-well tissue-culture plates filled with 3 mL of deionized water. The larvae were fed daily with TetraMin™ fish food (hatching day: 0.04 mg/larva, 1 day old: 0.05 mg/larva, 2 days old: 0.1 mg/larva, 3 days old: 0.2 mg/larva, 4: days old 0.4 mg/larva, 5 days old and older: 0.4 mg/larva). Two days after hatching half of the larvae received 104 spores of V. culicis (originating from a stock solution kept at 4°C) and the other half received a control solution of crushed, uninfected mosquitoes with the daily amount of food. The concentration was determined with a hemocytometer and a phase-contrast microscope. The number of spores was chosen because in previous experiments it led to close to 100% infection success but very little larval mortality. The pupae were individually placed into Falcon tubes and, once adults, provided with a cotton ball soaked with 10% sugar solution. Two days after emergence pairs of males and females were moved to 180-mL plastic cups for mating. All of the males were from the control treatment. Two, nine and 16 days later, females were allowed to take blood meals on GZ's arms for 10 minutes. The eggs were collected and stored in an incubator at standard lab conditions. The adults were then killed and stored in Eppendorf tubes at -80° C until the molecular analysis. Once mosquitoes were prepared for the extraction of the DNA (see below), we took 8 µl from the extraction tubes and confirmed the presence of V. culicis with a hemocytometer and a phase-contrast microscope. For further analysis, we haphazardly chose for each block and each treatment ten females that laid at least 40 eggs (over their three blood meals) and that were heterozygous for at least two microsatellites.

Offspring generation

We bleached the eggs of all families with 1% household bleach (MR4, Methods in Anopheles Research) to eliminate possible spores of the parasite. The larvae of each family were reared in a petri dish containing 100 mL of deionized water and were fed daily with TetraMin™ fish food (hatching day: 0.06 mg/larva, age 1: 0.08 mg/larva, age 2: 0.16 mg/larva, age 3: 0.32 mg/larva, age 4: 0.64 mg/larva, age 5 and older: 0.32 mg/larva). The pupae of each family were moved to a cage containing a 10% sugar solution. Once all the pupae had emerged, we killed the mosquitoes and stored them individually in Eppendorf tubes at a -80° C. An average of 19 offspring per family (4 to 33) were haphazardly chosen for the molecular analysis.

5.3.3 Molecular analysis

DNA Extraction

A different procedure was used for the parents and the offspring because of the different sample sizes of the offspring and the parental generations. For the offspring, we extracted total DNA with QIAGEN DNEasy® 96 Blood & Tissue Kit (plate extraction) following the protocol of the manufacturer. The samples were randomized, and each DNA extraction plate contained 95 mosquitoes and one negative

DNA extraction control (Ixodes ricinus tick). For the parental generation we extracted total DNA using the QIAGEN DNEasy® Blood & Tissue Kit (individual tubes).

PCR

PCR amplification of the selected microsatellites was performed in 96-well plates with a thermocycler Mastercycler (Eppendorf). The PCR mix for one reaction contained 5 µL 5X GoTaq® Reaction Buffer (Promega), 0.5 µL PCR Nucleotide Mix (10 mM of each dNTP) (Promega), 1 µL of each primer (10 µM), 0.2 µL GoTaq® G2 DNA Polymerase (Promega) and 2 µL DNA template. A final volume of 25 µL was reached with PCR-grade water. Each plate contained the DNA of 88 samples randomly assigned, 4 intra-plate replicates, 3 inter-plate replicates and 1 negative control.

The thermocycling conditions of the PCR amplification for microsatellites A, B, D and F included a denaturation step at 94 °C followed by 30 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s and 1 min of extension at 72°C, followed by 10 min final extension at 72°C. For microsatellites C and E, the annealing temperature was changed to 55° C and 51° C, respectively. The PCR products were sent to Microsynth AG for the capillary electrophoresis genotyping.

Genotyping and Recombination fraction

We used the program GeneMapper Software (version 4.1) to obtain the genotypes of the samples. We used CRI-MAP (version 2.507) to determine the recombination rate in each mother (i.e., the proportion of offspring that carried a combination of alleles that she did not have). 624 offspring were screened (252 for block 1 and 372 for block 2). The recombination rate for each of the 15 pairwise combinations of microsatellites was defined as the ratio of the number of recombinant offspring and the total number of offspring. Recombination between the loci A and F and between A and D of the uninfected treatment could not be detected with CRI-MAP (Figure 2).

5.3.4 Statistical analysis

We analysed the number of recombination and non-recombinant offspring per family with a GLME (generalized linear mixed effect model) with binomial errors. We included in the analysis the experimental block, and the infection status of the mother, the expected recombination rate between pairs of microsatellites and their interaction as fixed factors. We included the mother as a random factor to control for the multiple microsatellite pairs per mother. The expected recombination rate was obtained from a transformation of the genetic distance between pairs of microsatellites given in the literature (Severson et al. 2002; Timoshevskiy et al. 2013) with Kosambi's function, which corrects for interference and multiple crossing-overs that can occur at large genetic distances (Haldane 1919; Kosambi 1944; Huehn 2011). The transformation also takes into account that the probability of recombination between two loci increases with the distance between them (Sturtevant 1913). All statistical analyses were performed with R version 3.4.2 and the RStudio interface version 1.1.183. The

lme4 (Bates et al. 2014) and car (Fox & Weisberg 2011) packages were used for the mixed effect models.

5.4 Results

The recombination rates between pairs of microsatellites ranged from 0% to 50%. Infected mothers had higher recombination rates than uninfected ones in 10 out of the 13 pairs of microsatellites in which recombination was detected in both treatments (Figure 3). If there were a probability of 0.5 that infected individuals have a higher rate of recombination than uninfected ones, this or a more extreme pattern would occur with a probability of 0.046.

Observed recombination rate increased with expected recombination rate for uninfected and infected individuals (main effect of infection: χ^2=18.49, df=1, p<0.001), but in the infected individuals the recombination rate increased more steeply with expected recombination than in uninfected individuals (interaction infection*expected recombination: χ^2=3.89, df=12, p=0.02). Indeed, between the microsatellite couples A-B and A-C, infection nearly doubled the recombination rate. The experimental block had little impact on recombination rate (χ^2=2.75, df=12, p=0.15).

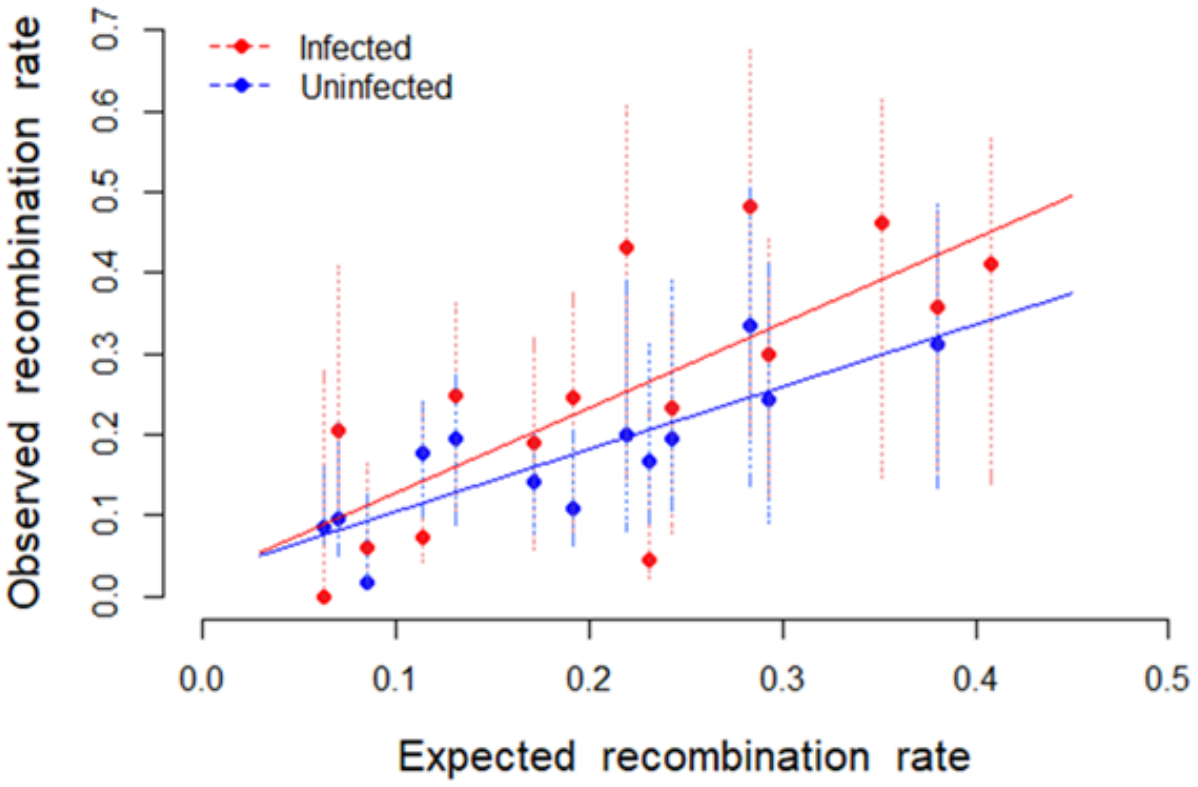

Figure 2 Observed recombination rate of microsporidian-infected and uninfected *Aedes aegypti* as a function of the expected recombination rate. Each red and blue circle (and the corresponding red and blue dashed lines), represents the mean ± 95% confidence interval of the proportion of recombinants in the screened offspring (observed recombination rate) per couple of microsatellites of the respectively infected and uninfected treatment. The red and blue solid lines represent the regression line for the infected and uninfected individuals.

5.5 Discussion

Our data indicate a phenotypically plastic increase of recombination rate of the yellow fever mosquito Ae. aegypti in response to infection by the microsporidian *V. culicis*.

Our study corroborates previous ones in animals and plants. Females of *Drosophila melanogaster* challenged with a variety of parasites plastically increased the proportion of recombinant offspring they produced (Singh et al. 2015). The increase in recombination rate was found with a Gram-positive bacterium, Gram-negative bacterium and a parasitic wasp. A similar response was found in the leaf tissues of *Arabidopsis thaliana* and *Nicotiana tabacum* after infection by *Peronospora parasitica* (Lucht et al. 2002; Kovalchuk et al. 2003). These results found with bacterial, fungal and animal parasites suggest that a plastic increase of recombination rate could be a general response to parasitism, although bacterial infection does not appear to increase recombination rate in house mice (Dumont et al. 2015).

The reason for this response is likely that increased recombination in infected individuals increases the diversity and the frequency of novel genotypes in their offspring. This may help infected individuals to protect their offspring against infection by the same parasite, if the success of the parasite depends on several genes of its host's genotype. As the parent's parasites have been able to overcome its genetic resistance, offspring with unfamiliar genetic combinations are expected to be more resistant against the same parasites. This idea underlies a version of the Red Queen Hypothesis, which states that the pressure imposed by parasites is responsible for the maintenance of sex and genetic recombination. Moreover, since the rate of recombination (Chinnici 1971; Kidwell 1972; Shaw 1972; Abdullah & Charlesworth 1974) and resistance against parasites (Koskela et al. 2002; Råberg et al. 2007; Mazé-Guilmo et al. 2014) are heritable traits that can respond to selection, strong parasite pressure can select for a higher rate of recombination (Peters & Lively 1999; Schmid-Hempel & Jokela 2002; Peters & Lively 2007; Salathé et al. 2007). Recombination rate may therefore evolve in response to parasites' selection and directly influence fitness-related traits in the host.

In addition to the experiment mentioned earlier, where the red flour beetle increased its recombination rate after several generations of coevolution with a microsporidian parasite (Fischer & Schmid-Hempel 2005; Kerstes et al. 2012), several studies support the idea that parasites select for greater recombination. Two examples are that sexual populations of the freshwater snail *Potamopyrgus antipodarum* are less frequently infected than asexual ones (Jokela et al. 2009; Vergara et al. 2014; Gibson et al. 2016), and that the coevolution between *Caenorhabditis elegans* and *Serratia marcescens* increases the level of outcrossing (and thus, presumably, genetic recombination) (Morran et al. 2009) and constrains the spread of self-fertilization (Slowinki et al. 2016). Our study expands on

these experiments by showing that recombination rate can change immediately and plastically rather than increasing evolutionarily over time.

Differently from previous work, we focused on microsatellites rather than visible mutant genetic markers (Singh et al. 2015), which themselves can have fitness costs (Mark & Zimmering 1977). In fact, the main goal of this research was to investigate variation in recombination rate due to parasite infection at global chromosomal level, without searching for associations with specific genes and mechanisms which would have require a more precise scale (Stevison et al. 2017).

To conclude, our study shows a link between parasitism and recombination rate, corroborating the role of parasites in the maintenance of sex. In contrast to most studies, however, ours emphasizes that the response of a host's recombination rate to infection can be plastic. This plastic response helps the host to protect its offspring against its parasites escaping the detrimental parasite pressure without directly involving immune or immune-related response and genes. Contrasting and combining the plastic and genetic effects of parasites on recombination and their consequences for genetic diversity will be critical to understand how host and parasite populations co-evolve. Furthermore, since our host is an important vector of many parasites of humans, our study suggests the relevance of theoretical and practical investigations of the impact of parasites on the recombination rate of *Ae. aegypti* and the potential for ecological and evolutionary feedbacks.

Chapter 6

General discussion

6.1 Summary of results

6.1.1 Chapter 2

In this chapter, we studied how co-infection, the availability of resources of the host and the timing of infection affected the transmission success and virulence of two parasites with conflicting mode of transmission. Our findings illustrate that the interactions between these factors highly influenced host-parasite dynamics. Overall, early infection resulted in higher transmission potential compared to late infection in either single or multiple infection. In accordance with the idea of competition for shared and limited resources, the parasite infecting first was able to better exploit the host. Food restriction increased virulence and juvenile mortality, and enhanced horizontal transmission among larvae, whereas the abundance of food increased the proportion of mosquitoes emerging and lead to the transmission from adult to larvae. The co-infection increased the potential for transmission of the strictly horizontal transmitted parasites in the juvenile stage of the host and resulted in higher virulence. In the adults, the potential transmission of the mixed-mode transmission parasite, which is a specific parasite to the host, was not affected by the competitor. When infecting first during co-infection, it imposed its vertical transmission and reduced the probability of horizontal transmission of the other parasite. Thus, its vertical transmission from adults to larvae was highly effective, no matter the competition. The co-infection in the adults led to higher virulence and reduced longevity. The latter was only true for the obligate horizontally transmitted parasite, as the longevity of the mosquitoes infected with the parasite presenting a mixed mode of transmission did not differ in single or multiple infection. The results emphasize that to better understand host-parasite evolution and diseases outcome, ecological and epidemiological interactions need to be considered.

6.1.2 Chapter 3

In the third chapter, we investigated the conditions influencing the trade-off between vertical and horizontal transmission, the resulting level of virulence and the consequences for the evolution of the transmission mode of a mixed-mode transmission parasite. We found that host's growth environment and genotype affected the transmission strategy of the parasite. The parasite invested and adjusted the transmission according to the given settings. In particular, it exhibited effective vertical transmission when the host was expected to achieve high fecundity and more horizontal transmission when the host had a slow rate of development. The results also show how the parasite switched in an adaptive way the transmission mode according to the genetic background of the host they were infecting. Indeed, hosts that permitted more vertical transmission permitted less horizontal transmission and vice versa, such a trade-off was particularly clear under stressful host conditions. Our results show that environmental variation may affect parasite transmission strategy and contribute to the important concept of transmission mode evolution bringing empirical evidence of the mechanisms

and the trade-offs leading to different evolutionary pathways in transmission modes. Moreover, this represents a rare case showing that the genetic variation in the host rather than in the parasite can considerably influence the epidemiology or drive the evolution of the transmission mode in the parasite.

6.1.3 Chapter 4

The ecological conditions may strongly influence the transmission of the parasites. In this chapter, we assessed with an evolutionary experiment whether the vertical and horizontal transmission component and the relative virulence of a mixed-mode transmission parasite responded to restriction opportunities represented by the environmental conditions of the host. The results show that after few generations of evolution under different host's resource availability, the parasite responded to selection by modifying the relative importance of the two transmission components, and by affecting host's life history traits (juvenile development, size, and longevity). Low resources during evolution lead to an increase in the potential for vertical transmission, in larger host's body size and increased longevity. The fluctuation of resources at each generation favoured the potential for horizontal transmission and delayed juvenile development, whereas the evolution under constant availability of high resources had no effect. The results confirm the potential role of the environment in determine evolutionary transitions, and how the resulting selective pressure of the environmental conditions may push the parasite towards different evolutionary trajectories, with relevant consequences for host-parasite interactions. Further, this experiment reveals the presence of genetic variation for transmission mode in a eukaryote parasite presenting a developmentally-fixed program. Our findings contribute with direct evidence and empirically show a parasite's evolution in response to the environmental conditions.

6.1.4 Chapter 5

In the fifth chapter, we explored a potential genetic defence of the host. We found that the infected females increased their recombination rate, and that they were more likely to produce recombinant offspring than the uninfected ones. Thus, the females were able to diversify their progeny via a plastic response to an infection. Genetic recombination may represent a defence mechanism for the host to protect the offspring against its parasites. Since hosts are favoured by producing offspring with rare and unusual genetic combinations, selection will tend to maintain a high recombination rate in populations where parasites are common. This is in accordance with the version of the Red Queen Hypothesis affirming that parasites' pressure is responsible for the maintenance of sexual reproduction and recombination. The results reinforce this idea, confirming that parasites have a part in the maintenance of sex. Further, the study expands the classical view of the theory emphasizing that the increase in recombination rate can be plastic rather than an evolutionary phenomenon. To our knowledge, this is the first study showing how a parasitic infection increases host's recombination rate

in animals, without the use phenotypic markers to measure recombination. These results may have important consequences for the genetic diversity and the adaptability of host and parasite populations, and how these may co-evolve.

6.2 Further perspectives

6.2.1 Chapter 2

In the second chapter we highlighted the importance of being the first parasite to infect the host in the context of multiple infections. A further step could be the evaluation of the within-host dynamic of the two competing parasites by killing the host at fixed time-points and measuring the spore load. This would permit the full-scale analysis of the growth of the parasites during the juvenile and adult stage of the host, and a more exhaustive determination of their transmission strategy during co-infection.

6.2.2 Chapter 3-4

How variation and fluctuation of the environmental conditions influence host-parasite interactions remains a fundamental open question. Spatial and temporal heterogeneity might alter epidemiology through changes in host density, probability of transmission and virulence (Anderson & May 1978; Wolinska & King 2009), and it could be further investigated. For example, host's background mortality, interspecific larval competition, predation, or the availability and quality of the oviposition site may be important ecological factors that could be manipulated to study the conditions favouring horizontal or vertical transmission. Another possible approach to test the theoretical assumptions of how parasite's transmission may evolve is experimental evolution (Kawecki et al. 2012). In our case the evolutionary experiment has shown the presence of genetic variation in the transmission of a parasite with a mixed mode of transmission and with fixed stages of development, and how this can rapidly respond to restriction opportunities represented by host's resources. The long-term experiment might be reproduced for a longer period of time, also considering using spores harvested from horizontal infected larvae dying before adulthood or from the vertically infected larvae dying at different time point (different within host-growth of the parasite). A further step could be to conduct a GWAS on the parasite lines obtained after evolution. For the genome of *Edhazardia aedis* has already been sequenced (Desjardin et al. 2015), this could permit to identity the genes involved in the evolutionary transition of the transmission mode and the associated virulence. In addition, considering the effect of host's genetic background on the trade-off between vertical and horizontal transmission, an experiment to test the evolution of the transmission mode with the parasite infecting different host's families for several generations could be set up.

6.2.3 Chapter 5

In chapter five I described a plastic increase in recombination rate of a mosquito species after infection. Together with the work of Singh et al. 2015, this is one of the first example showing such a response to a parasitic challenge in the host. The implication for the genetic diversity of host's and parasite's populations could be theoretically analysed, particularly considering that our host is an important vector of many human viruses. This experiment could be implemented by adding new microsatellites marker or using SNPs (single nucleotide polymorphisms) to provide a finer-scale resolution of recombination (Stevison et al. 2017). A next step could be to target specific genes instead of using neutral molecular marker as microsatellites. The best candidates would probably be the immune or immune related genes, as these are directly involved in a host's defence against parasites. These genes are highly variable and often represent hot-spots of recombination (Sommer 2015), it would be interesting to test whether an infection lead to an additional increase of their recombination rate. Further, associations with particular genes could be considered to find specific mechanisms which may also be represented by epigenetic effects. This would allow to explore how different regions of the genome differently respond to parasite infection and by which measures. Finally, the resistance and tolerance of the offspring to an infection with the same parasite could be measured to couple the increased recombination rate in the mothers with the fitness of the descendants.

6.3 Conclusion

This work has covered several relevant topics of evolutionary ecology to gain a better comprehension of the forces that determine host's and parasite's evolution. The thesis has globally shown that the co-evolutionary dynamics between host and parasite highly depend on the ecological conditions, and how these may affect many aspects of host-parasite interactions including life history evolution, virulence and transmission. The results here presented have implications for the future search in the field of parasitology and may help to predict the evolutionary trajectories of the parasites and their management. We are far from an exhaustive understanding of host-parasite associations in a constant varying environment and many questions remain, it is therefore essential to explore and integrate host-parasite interactions in an ecological context.

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