EDUARDO CARLOS COSTANTIN

AN INSECT-PARASITIC FUNGUS SYSTEM TO STUDY COINFECTIONS

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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ABSTRACT

COSTANTIN, Eduardo Carlos, D.Sc., Universidade Federal de Viçosa, April, 2024. **An insect-parasitic fungus system to study coinfections**. Advisor: Simon Luke Elliot. Co-advisors: Kenneth Wilson and Sheena Cotter.

Coinfections occur when two or more parasites concurrently infect the same host, and they are a widespread phenomenon in nature. With significant consequences for host-parasite interactions, coinfections can dramatically alter the severity and epidemiology of a disease, the fitness of the organisms involved, and the evolution of the parasites' virulence. In this thesis, we propose a study system to investigate coinfections, their outcomes and their environmental modulators. This system is composed of two parasitic fungi – *Beauveria bassiana* and *Metarhizium anisopliae* – and the coleopteran host *Tenebrio molitor.* These fungi have similar infection mechanisms and host exploitation strategies, allowing us to investigate coinfections while diminishing the influences of parasite identity and host-exploitation strategy as variables. In the first chapter, we establish the study system and investigate the effects of characteristics of infection on coinfection outcomes. We find that coinfections did not change virulence towards the host, and when both fungi successfully sporulated on the host's body, the number of spores produced was drastically reduced. We also showed that the location of infection changed the percentage of hosts colonized for both fungi and that the first parasite to arrive dominated the infection process. In the second chapter, we explore the effect of host nutrition on single and coinfections. Host diet has limited impacts on coinfection and parasite fitness, but influences host mortality. A high protein diet is a better substrate to fight fungal infections compared to a high-carbohydrate diet, contradicting prior evidence found in hemimetabolous insects. In the third chapter, we investigate the role of temperature. Higher temperatures diminish the dominance of the most competitive fungus, *Beauveria.* On the host side, higher mortality occurred within the *optimum* temperature range for the parasite (~26°C). Our results support the adoption of this model system to study coinfections. This system have allowed us to test the effect of environmental factors and variables related to the process of infection on coinfection. This knowledge can be used to understand the dynamics of coinfection, and to identify variables that could be considered in

other biological systems. Additionally, elucidating the interaction patterns between *Beauveria bassiana* and *Metarhizium anisopliae* will contribute to increasing the efficiency of entomopathogenic fungi in agroecosytems.

Keywords: Coinfections; Host-Parasite Interactions; Nutrition; Temperature; Biological control.

RESUMO

COSTANTIN, Eduardo Carlos, D.Sc., Universidade Federal de Viçosa, abril de 2024. **Um sistema de estudo composto por fungos parasitas de insetos para compreender as coinfecções.** Orientador: Simon Luke Elliot. Co-orientadores: Kenneth Wilson e Sheena Cotter.

As coinfecções ocorrem quando dois ou mais parasitas infectam o mesmo hospedeiro de forma concomitante e são um fenômeno frequente na natureza. Com significantes consequências para a interação parasita-hospedeiro, elas podem alterar dramaticamente a severidade e epidemiologia das doenças, o fitness dos organismos envolvidos, e a evolução da virulência dos parasitas. Nesta tese, estamos propondo um sistema de estudo para investigar as coinfecções, suas consequências para as espécies envolvidas e seus moduladores ambientais. Esse sistema é composto por dois parasitas fúngicos – *Beauveria bassiana* e *Metarhizium anisopliae* – e o hospedeiro *Tenebrio molitor.* Esses fungos possuem mecanismos de infecção e exploração do hospedeiro similares, nos permitindo estudar as coinfecções e diminuir a influência das identidades dos parasitas e da forma de exploração dos hospedeiros como variáveis biológicas que influenciam os seus resultados. No primeiro capítulo, nós estabelecemos o sistema de estudo e investigamos os efeitos das características da infecção no resultado das coinfecções. Nossos resultados mostraram que as coinfecções não alteram a virulência para o hospedeiro, e quando ambos os fungos esporulam no cadáver do inseto, o número de esporos produzidos foi drasticamente reduzido. Nós também demonstramos que a localização da infecção altera o percentual de hospedeiros colonizados pelos dois fungos e que o primeiro parasita a chegar no hospedeiro dominou o processo infecção. No segundo capítulo, nós exploramos o efeito da nutrição do hospedeiro nas infecções simples e coinfecções. A dieta do hospedeiro teve um impacto limitado no resultado da coinfecção e no fitness dos parasitas, mas influenciou a mortalidade do hospedeiro. A dieta rica em proteína se provou ser um melhor substrato para combater infecções fúngicas comparadas com a dieta com maior quantidade de carboidratos. Isso contradiz evidências prévias do efeito da dieta em insetos hemimetábolos. No terceiro capítulo, investigamos o efeito da temperatura em nosso sistema. O aumento da temperatura diminuiu a prevalência do fungo dominante, *Beauveria.* Já com relação ao hospedeiro, uma maior mortalidade foi observada dentro da temperatura ótima

para o desenvolvimento dos parasitas (~26°C). Nossos resultados suportam a adoção desse modelo de estudo para investigar as coinfecções. O sistema nos permitiu testar o efeito das variáveis ambientais e das características da infecção nas coinfecções. Esse conhecimento pode ser usado para entender as dinâmicas das coinfecções, e identificar variáveis que podem ser usadas em outros sistemas biológicos. Além disso, elucidar os padrões de interação entre *Beauveria bassiana* e *Metarhizium anisopliae* pode contribuir para aumentar a eficiência dos fungos entomopatogênicos na agricultura.

Keywords: Coinfecções; Interação parasita-hospedeiro; Nutrição; Temperatura; Controle biológico.

SUMMARY

GENERAL INTRODUCTION

1. Host-parasite interactions

Hosts and parasites constantly interact in natural and anthropogenic habitats. This interaction is harmful to hosts and this harm can be observed as disease, which is the clinical manifestation of the damage inflicted by the parasites (Casadevall and Pirfoski, 2000). Diseases caused by parasites are ubiquitous and can severely impact hosts at an individual and population level. For example, the fungus *Batrachochytrium dendrobatidis* can infect at least 520 species of amphibians (Olson et al., 2013) and has already caused several species extinctions (Cheng et al., 2011). In *Homo sapiens*, viral pandemics are also responsible for the infection of millions of humans worldwide (Roychoudhury et al., 2020). These examples are among the most visible effects of host-parasite interactions, but today it is widely recognized that these interactions are of fundamental importance both in structuring biological communities and in mankind's efforts to manage natural resources (Lafferty et al., 2006).

Traditionally, research on host-parasite interactions has focused on the relationship between one parasite and one host. This methodology has been useful to understand the life cycle of parasites, their negative effects on host fitness and their transmissibility. However, hosts are exposed to multiple potential parasites during their lives, and these infections can occur concurrently. In fact, since the late 1990s, several researchers have highlighted the role of coinfections in animals and plants (Petney & Andrews, 1998; Cox, 2001), suggesting that this is likely to be the rule rather than the exception in nature. Therefore, the investigation of one parasite - one host relationships may be a gross simplification of what occurs in most biological systems. Despite this, most research on such systems, whether pure or applied, still considers such two-way interactions and there has been little empirical work even on systems where a host is infected by two parasites that can be considered to have general relevance.

2. Coinfections

Coinfections occur when two or more species of parasites - or different genotypes of the same species - coexist in a single host (Cox, 2001). In this thesis, we are using the term parasite in a broad sense, which encompasses all organisms that live at the expense of another organism (host). Parasitic helminths, nematodes, and arthropods are considered macroparasites here, while parasitic fungi, nematodes, protozoa, bacteria and viruses are microparasites.

Coinfections have been recorded in different taxa, including humans, plants, birds, reptiles, amphibians, and fish; and have been the subject of revisions in some of these instances (Griffiths et al., 2011; Tollenaere et al., 2016). They can dramatically alter the severity and epidemiology of the disease (Hoarau et al., 2020), the fitness of the organisms involved (Redman et al., 2016), and the evolution of the parasites' virulence (Van Baalen and Sabelis, 1995; Alizon and Van Baalen, 2008). Historically, coinfections have been investigated extensively in vertebrates infected with helminthic parasites (Cox, 2001). Currently, these systems still comprise a significant part of these studies, but coinfections in hosts such as plants and insects by other groups of microparasites (eg. viruses and fungi) are gaining recognition. For example, in the field of plant pathology, the virus-virus interaction is well-documented in several plant species, but its epidemiological and evolutionary consequences are just beginning to be understood (Moreno and Moya, 2019).

One of the main determinants of coinfection outcomes is the nature of parasite-parasite interactions, also known as within-host interaction. These can occur by direct interactions or indirectly, through an intermediary, such as the host's resources or immune system.

3. Parasite-Parasite interactions in coinfections

Parasites can interact directly or indirectly in coinfections; in both cases the effects of one parasite on the other can be negative or positive. Positive direct interactions commonly occur through cooperation between parasites in the production of public goods. This is also best-known from bacteria, such as those that produce siderophores (West and Buckling, 2003). They can also occur through mechanical facilitation by one parasite, which promotes the colonization of the co-infective organism. This can be seen in wounds caused by the ectoparasitic crustacean *Argulus coregoni* on the skin and mucous membranes of a fish (*Oncorhynchus mykiss*); such wounds facilitate the entry of a microparasitic bacterium that causes columnariasis (Bandilla et al., 2006), a disease that can severely impact aquaculture. Negative direct interactions occur when the physical presence or metabolites produced by one parasite affect the other. This is well-known in bacteria: for example, *Bacillus subtilis* is able to inhibit growth of the human pathogen *Staphylococcus aureus* through the antibiotic metabolite surfactin (Gonzalez et al., 2011).

Indirect interactions will always occur through an "intermediary", which can be the host as a resource to be consumed, or through its immune system. One type of positive

indirect interaction is immunosuppression, in which one of the parasites facilitates the colonization of the other organism by weakening the host's immune system. For example, the parasitic mite *Varroa destructor* is capable of destabilizing the relationship between bees and the Deformed Wing Virus (DWV). This mite can lead to an immunosuppression syndrome via downregulation of the NF-kB transcription factor (Nazzi et al., 2012), which in turn enhances colony susceptibility to the virus. This interaction gained notoriety for causing the loss of colonies worldwide (Kielmanowicz et al., 2015). On the other hand, indirect negative interactions between parasites are commonly described. This can occur through competition for host resources as has been demonstrated in several coinfective systems (eg. Kinnula et al., 2017). As a rule, competition for a limited resource negatively affects individuals by decreasing their size, fecundity or life span. Another example of negative indirect interaction is cross-immunity, which occurs when infection by one parasite promotes an efficient immune response against another. There is evidence of cross-immunity between species of the genus *Plasmodium*, in which the avirulent species (*Plasmodium vevax*) offers protection against a virulent species which causes malaria in humans, (*Plasmodium falciparum*) (Maitland et al., 1997).

This myriad of interactions between coinfective parasite could lead to many different coinfection outcomes, which could result in fitness consequences for hosts and parasites. The understanding of this fitness impact in coinfective relationships is perhaps the most basic and important step to start to disentangle coinfections.

4. Impacts of coinfection on host and parasite fitness

Coinfections may have negative, positive or no impacts on host and parasite life-history and fitness. Regarding the host side, virulence - defined here as the reduction on host fitness due to infection (Read, 1994) - can be increased, remain unchanged or be reduced by the presence of another coinfecting parasite. In the armyworm (*Spodoptera exempta*), coinfection with different variants of *Spodoptera exempta nucleopolyhedrovirus* (SpexNPV) kills the host 2.7 times faster than infection with a single variant (Redman et al., 2016). In contrast, in the model organism *Daphnia magna*, coinfection with different *Pasteuria ramosa* genotypes resulted in a mild negative effect on female fecundity when compared to single infections (Ben-ami et al., 2008). The synergistic or antagonistic effect on virulence will depend on the strategies used by each parasite to exploit its host, on the niche occupied by each of them

(Pedersen and Fenton, 2007), and on degree of relatedness of these parasites (Buckling et al., 2008).

The life-history of parasites is also impacted by coinfections. However, this impact is less understood, especially because the traits of these organisms such as growth and propagule production are difficult to estimate (Pariaud, 2009). In most cases, sophisticated and expensive molecular techniques, such as quantitative PCR, are required to estimate parasite population sizes or their fitness. Therefore, biological systems in which parasite fitness can be clearly assessed are extremely relevant. Understanding parasitic fitness in coinfections is essential to understand the impact on epidemiology and to develop interventions and management strategies in relevant diseases to human/animal and plant health.

The course of coinfection and its impact on both parasite and host fitness can be influenced by the surrounding dynamic environment. Factors such as temperature and host nutrition, which are known to affect one host-one parasite relationships, may also be relevant to the outcomes of coinfections.

5. Characteristics of infections and environmental modulators

Comparing the outcomes of single and coinfections under controlled conditions is the first step to understand multiple parasite infections. However, host-parasite relationships occur in a variable environment, in which temperature and availability or quality of food resources can vary considerably. In addition, the characteristics of infections such as arrival order and the time between infection of the first and the second parasites are also dynamic. Therefore, the environmental context in which coinfections are inserted must be considered, since they are capable of radically changing their results.

The arrival order and the time span between the infection of one parasite and the other are quite variable. Despite this, studies in the area predominantly inoculate the parasites simultaneously and at the same point of infection in their hosts. As pointed out by Karvonen et al., (2019), situations like these are less likely to occur than those in which parasites are inoculated, for example, at different periods of the host's life, given that these relationships are dynamic. Some studies have altered the order of arrival of parasites or spaced the infection in different intervals (de Roode et al., 2005; Natsopoulou et al., 2015; Zilio and Koella, 2020) and they have obtained interesting results. A classical example of the importance of parasite arrival order was found by Ezenwa et al., 2010. The African buffalo (*Syncerus caffer*) when

first infected by nematodes, decreases the number of Th1 immune cells (responsible for an effective response against intracellular parasites, such as viruses and bacteria) and increases the number of Th2 immune cells (which produce immune responses against macroparasites, such as nematodes). This imbalance favors the colonization of the microparasite *Bacterium bovis*, the causal agent of bovine tuberculosis, a disease that causes serious damage to the lungs and liver of infected animals.

The effects of being the first to arrive (priority effects) could be relevant to the outcome of coinfections not only in an individual level, but also can alter parasite community structure (Halliday et al., 2020) and ultimately influences natural epidemics (Clay et al., 2020). The biological mechanisms behind the within-host priority effects in community ecology of parasites will depend of the identity of parasite and how they interact with host immune response (Pedersen and Fenton, 2007). For example, beneficial priority effects are expected to be pronounced when the competing species exhibit high within-host niche overlap (Ishii et al., 2002) or the first invader elicits a strong immune response against the second (Singh et al., 1999).

In addition to the characteristics of the infection, environmental conditions can also alter the outcome of coinfections. Temperature is known to alter the course of one parasite - one host relationships (Elliot et al., 2002) and also impact the fitness of the organisms involved. However, in coinfection, the role of temperature is less understood. Fargues and Bon (2004) reported that different strains of *Isaria* (=*Paecilomyces*) *fumosoroseus*, an insect pathogen, competitively excluded each other across most temperature gradients. However, between 20-25°C they were able to coexist in the same host. Inglis et al. (1997) and Malakar et al. (1999) also reported the role of temperature in coinfections, when synergistic effects on host mortality became apparent at higher temperatures. Thus, a temperature gradient may influence the outcome of coinfections and act as a selection pressure for the population of entomopathogenic fungi (Bidochka et al., 2002). Furthermore, a varying temperature can alter priority effects and facilitates species coexistence rather than exclusion (Tuker and Fukami, 2014). This interaction between temperature and priority effects was observed in the establishment of a community of microbes that colonizes nectar, but it could also affects the community of parasites.

Host nutrition is another relevant factor in the dynamics of coinfections. The quantity and quality of food found by living organisms in the course of their life is a challenge for their

survival. In addition to the already expected effect on growth, life span and reproduction, nutrition also impacts the organism's ability to face and resist diseases (Ponton et al., 2020). Parasites consume a common resource in infections: the host. Thus, changes in host nutrition and, consequently, in host metabolism can lead to different patterns of interaction between parasites, especially if the nutritional resources of one of them are different from the other (Ezenwa, 2021). In rodent malaria (a model system), the interaction between two different strains (one virulent and one avirulent) of *Plasmodium chabaudi* is mediated by the nutrient folate, which is obtained from the host's diet. Consequently, differences in dietary folate acquisition have a major impact on the course of the disease (Wale et al., 2017). In invertebrates, changes in diet quantity modify the outcome of coinfection but this depends on the biological system studied (Fellous and Koella, 2010; Duncan et al., 2015; Deschodt and Cory, 2022). However, no major conclusions or generalizations can yet be drawn about the effect of nutrition in coinfection and more studies are needed to understand the underlying mechanisms.

Despite the characteristics of infections, temperature, and diet being theoretically recognized as modulators of coinfections, empirical studies recurrently neglect these variables. One of the main reasons why this occurs is the experimental and methodological difficulty in considering these variables in studies that are already complex as they encompass different species or genotypes. However, to better understand coinfections and their consequences and to develop disease management strategies, it is necessary to take into account the temporal and environmental variability that permeates these interactions.

6. Study system and thesis overview

There are still many gaps in basic and applied research that covers coinfections. For example, they are usually studied from a host-centered perspective and environmental variables are neglected when studying them. This thesis aims to address part of these problems using a system composed of two entomopathogenic fungi – *Beauveria bassiana* and *Metarhizium anisopliae* - belonging to the same taxonomic order of fungi (Hypocreales). These fungi have similar infection mechanisms and host exploitation strategy, which allows us to experimentally focus on the effects of the environment (temperature and diet), and the characteristics of the infection, such as the order of arrival of the parasite, time between infections, and site of infection. In addition, *B. bassiana* and *M. anisopliae* have different

spore colors - white and green, respectively - and this makes their differentiation possible without the use of sophisticated and expensive molecular techniques. This practical aspect of spore differentiation will allow us to easily quantify parasite fitness in coinfections. The use of these parasites also has direct practical relevance. The two selected fungi are the most used in biological control of insects in Brazil and the world, including in mixtures, and understanding how they interact will help to develop more rational pest control strategies, aiming a sustainable agriculture. As a host, we selected the beetle *Tenebrio molitor*. This insect has several advantages for this research, as its colony is easy to maintain in the laboratory, it is also widely used for studies on insect pathology and is currently considered a food source in Europe (which means that the study of the pathogens of this insect has practical relevance).

This thesis has three chapters. In Chapter One, we established our model system and tested characteristics of infection that could change the outcome of coinfections. The isolates of *Beauveria* and *Metarhizium* were selected and virulence tests were performed. We then assessed the consequences of coinfections for the host and for the fungal parasites in baseline conditions, in which the parasites were inoculated at the same time and in the same place on the host's body. We performed experiments varying the location of parasite infection on host body, order of arrival and time between infections to estimate the impacts of these variables on coinfections. The main results showed that coinfection between *Beauveria* and *Metarhizium* did not affect virulence towards the host, but parasite fitness was significantly impaired when both fungi sporulated on host's body. Additionally, parasite inoculation in different locations on the body increased the number of dead hosts showing signs of both fungi, compared to when parasites were inoculated in the same place. Furthermore, the first parasite to arrive dominated most infections.

In Chapter Two, we investigated the effects of host nutrition on coinfections. Nutrition is a key modulator of host-parasite interactions with impacts on the fitness of hosts and parasites. However, its effects on fungal infections remains largely unexplored. We used three chemically-defined diets varying their content of protein and carbohydrate to perform the experiments and assess the effects of macronutrients on single and coinfections with entomopathogenic fungi. We found a limited impact of diet quality on coinfections and parasite fitness. However, hosts fed on a high-carbohydrate diet had higher mortality rates compared to the balanced and a high-protein diets.

Finally, in Chapter Three we investigated the impacts of environmental temperature in our system. Temperature influences the majority of the biological process of ectothermic hosts, and the relationship between hosts and parasites. Using a combination of *in-vitro* and *in-vivo* experiments, the patterns of interaction between both parasites and the host were evaluated across a temperature gradient. We demonstrated that temperature changed the interaction between the parasites *in-vitro* and this might have impacted the outcome of coinfection *in-vivo.*

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CHAPTER I

Insect-parasitic fungi as a model system to investigate coinfections

Insect-parasitic fungi as a model system to investigate coinfections

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ABSTRACT

Living organisms are often infected simultaneously by different parasites. The outcomes of such coinfections can be largely based on the identity of the parasites involved, which renders investigation of other variables difficult. To address this problem, we report here the establishment of a model system composed of two entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to study coinfections. These two fungi interact in a similar fashion with the host and this allows us to examine coinfections experimentally while largely excluding parasite identity and host-exploitation strategy as variables. Coinfection between both fungi in standard conditions – inoculated at the same time and in the same place - did not enhance virulence towards the host, larvae of the beetle *Tenebrio molitor.* In this scenario, *B. bassiana* was the dominant fungus in 60% of infected larvae, *versus M. anisopliae* in 12%, and both parasites emerging in 28%. We then tested if intrinsic aspects of infection could modify this outcome, beginning with the location of infection. When both fungi infected the host in separate locations of its body, the percentage of coinfected larvae and the number of spores produced by the fungi increased. Order of inoculation also had an effect on coinfection, with the first parasite to arrive dominating the host, even for *Metarhizium* which proved to be a weak competitor in simultaneous infection. However, the time lag between infections did not alter this within-host priority effect. Our findings support the use of the proposed system to study coinfections since we can investigate a wide range of environmental and infection factors, including neglected ones, that can alter host and parasite fitness. We intend to generate a body of theory that also can be used in other biological systems that investigate coinfections. These results may also inform the use of these fungi as biocontrol agents in agriculture, given they are often used in conjunction.

1. INTRODUCTION

Host-parasite interactions have been studied extensively in the laboratory, allowing for controlled conditions in which specific variables can be examined experimentally. The challenge then is to add realism to increase relevance to the natural world. We know, for example, that variations in temperature and nutrition can strongly influence host-parasite interactions – incorporating these factors in the design of experiments can inform how infections may play out in the wild (e.g. Elliot et al., 2002; Miller and Cotter, 2018). Similarly, hosts often encounter multiple parasites, resulting in coinfections, which is when two or more parasite species or genotypes co-occur in the host simultaneously (Petney and Andrews, 1998; Cox, 2001).

Coinfections are the norm in nature and might influence various biological (Redmand et al., 2016), disease-related (Hoarau et al., 2020) and evolutionary features (van Baalen and Sabelis, 1995). As with single infections, coinfections are modulated by infection-specific and external variables, yet they are usually investigated in simplified environments where those factors are neglected (but see Zilio and Koella, 2020; Deschodt and Cory, 2022). The establishment of a model system to investigate these factors should help with both practical and theoretical aspects of animal or plant disease management.

It is salient to consider what factors could affect the progress of coinfections and so what could be of interest to study in a model system. Some factors may be specific to how the infections themselves occur. For example, the parasites may initiate the infection process in the same or in different locations in the host. If they do this in the same location, this could enhance the chances of early negative interactions if they compete from the moment that they arrive. These negative interactions could be direct, when parasites are close to each other and compete for space (Pedersen and Fenton, 2007); or indirect through competition for host resources, as has been demonstrated in several coinfective systems (eg. Kinnula et al., 2017). Early competition may favor one species and lead to competitive exclusion of the other. We know that niche occupation inside a host can impact the outcome of coinfections (Lello et al., 2018). Indeed, parasites that occupy different niches within a host (these parasites are usually only distantly related to one another) are more likely to coexist (Rynkiewicz et al. 2015). However, is still unclear how different location of infection by parasites that will occupy the same niche inside a host might affect the outcome of coinfections.

Another factor intrinsic to the infections themselves is the order in which the parasites infect the host and the time that elapses between the first and second infection. Parasites are more likely to infect a host sequentially rather than simultaneously (Karvonen et al., 2019); where infections are sequential, this can modify the outcome of coinfections and consequently affect host and parasite fitness. Sequential coinfections by an intestinal protozoan and a fungus can be less virulent than simultaneous coinfection in the waterflea *Daphnia magna* (Lohr et al., 2010); but more virulent between two viruses in the pepper *Capsicum annum* (Kim et al., 2010). Sequential infection can also affect parasite competitive success: de Roode et al. (2005) showed that the less competitive *Plasmodium chabaudi* parasite in coinfection can be competitively superior if it infects the host first in the rodent malaria system. This temporal aspect has been studied in *D. magna* (Lohr et al., 2010; Manzi et al., 2021), some plant species (Marchetto and Power, 2018) and mosquitoes (Zilio and Koella, 2020) as reported above, but is still overlooked in most animal systems.

Here, we undertook to establish a model system in which to study coinfection. We selected as parasites two entomopathogenic fungi: *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi have different spore colors – white and green, respectively – which makes their differentiation possible visually under a stereomicroscope. This is far simpler than molecular techniques, such as real-time PCR. Furthermore, these fungi belong to the same taxon (Hypocreales) and are both obligate killers (*sensu* Ebert and Weisser 1997) with similar mechanisms of infection and host exploitation (see Hajek and St. Leger, 1994). These features allow us to focus experimentally on factors that do not depend heavily on basic biological aspects of disparate organisms (versus coinfection with a virus and a nematode as an arbitrary example). For a host, we selected the beetle *Tenebrio molitor* L. which has several advantages. Firstly, it is reared commercially and is easy to obtain. Secondly, colonies are easy to maintain in the laboratory and this insect is widely used for studies on insect pathology. Finally, its larval stage is increasingly used as a food stuff for humans and other animals (Grau et al., 2017), so study of its pathogens is of practical relevance.

As a first step, we focus on intrinsic aspects of infection, rather than external environmental variables. We first selected one isolate of each fungus species with similar virulences. Then, we determined the outcome of coinfection when both were inoculated simultaneously and in the same location – as is done in most studies in this area using parasites with the same infection mode. Finally, we investigated how the location of infection,

order of parasite arrival, and the time between infections modify the observed pattern. Our focus is on the fitness of the parasites, rather than how coinfection affects host mortality or virulence (see review by Tinsley et al. in prep). For this, we adopt a broader ecological approach wherein hosts can be be thought of as an ecosystem in which multiple parasites can interact (Pedersen and Fenton, 2007; Rynkiewicz et al., 2015).

By experimentally varying the location of infection of our host insect by *Beauveria* and *Metarhizium* (in distant parts of its body), we hypothesize that competition between parasites will be delayed, increasing the chances of successful establishment of both fungi. This is not dissimilar to the general ecological principle that spatial distribution of species affects competition and allows for more coexistence of strains or species (Raventós et al., 2010; Detto and Landau, 2016). Then, To test if order and time between inoculations will affect coinfections, here we varied the order in which each fungus infects the host and we spaced the first and second infections in intervals of 12 hours up to a total of 48 hours between the first and the second infection. We predicted that the first parasite to arrive will have an advantage colonizing the host and that this advantage will be more pronounced the later the second parasite enters in the system. As with the question of space above, sequential colonization of a host is very similar to the general ecological principle that the order in which organisms can colonize a habitat can determine their relative success in doing so (Shorrocks and Bingley, 1994).

2. MATERIAL AND METHODS

2.1 Insect colony

Larvae of *Tenebrio molitor* were reared in opaque plastic boxes (21×17×9 cm) with a ventilated lid at room temperature 25±1°C, 12:12 L:D photocycle, and 65 ± 10% relative humidity. Larvae were supplied *ad libitum* with a diet composed of a mix of oat bran, wheat fiber and wheat bran (1:1:1). Five slices of carrot were provided every 4 days. Boxes with larvae were checked twice a week and individuals which had pupated were manually transferred to new plastic boxes and covered with a sheet of A4 paper to allow emerged adults to hide. After adult emergence, they were transferred and maintained in plastic containers (41×26×7 cm) with the same diet. Once a month, newly emerged larvae and eggs were collected.

2.2 Fungal suspensions

All fungal isolates used in this study were obtained from soil from coffee fields in Minas Gerais, Brazil, using the insect bait method (Zimmermann, 1986; Moreira et al., 2019). To prepare fungal suspensions, we flooded sporulating fungal cultures cultivated on PDA (Potato Dextrose Agar) plates with 10ml of 0.01% Tween 80 and scraped the spores with a Drigalski spatula. The suspensions were then stirred for 1 min and filtered using sterile gauze. This procedure allowed the separation of spores from hyphal fragments. Spore concentrations were estimated in a Neubauer Improved Chamber and adjusted to the desired concentration through serial dilutions. The suspensions were stored at 5°C and spore viabilities were determined one day before the experiment. For this, an aliquot of 100 µl of the suspension was individually plated on BDA culture medium and incubated at 25°C for 18 h. Suspensions were only used if they reached 95% germination.

2.3 Virulence bioassay for isolate and dose choice

One isolate of *Beauveria bassiana* (B0265) and two isolates of *Metarhizium anisopliae (*S2107 and M0122) were selected from the Insect-Microbe Interactions Laboratory (LIIM) isolate bank to perform a dose-response experiment in order to compare virulences. Three *Beauveria* isolates presented similar LT50s (Median Lethal Time) in preliminary tests, thus we selected only one to perform the virulence bioassay. Fungal suspensions were obtained as described in 2.2. Stock concentrations were estimated using a Neubauer Improved Chamber

and ten-fold serial dilutions were prepared and adjusted to the following doses: 1×10^4, 1×10^5, 1×10^6, and 1×10^7 spores ml^{-1} for all selected isolates. In total, we had 12 treatments plus the control group which was inoculated with blank 0.01% Tween 80. For each isolate × dose combination, 20 *T. molitor* larvae (100-140mg) were used in a factorial design. One microliter of the suspension droplet was topically inoculated on to the second thoracic segment of *T. molitor* larvae using a 2µl manual pipette. Each insect was observed until the liquid component of the suspension had been absorbed completely by the insect's cuticle. If the suspension came into contact with the Petri dish or the filter paper, the larva was replaced as we felt the total dose may have been reduced in these cases. After inoculation, each larva was placed in an individualized 15 × 60 mm Petri dish, lined with filter paper and moistened with 200µl of sterile water. The plates were sealed with cling film and placed in an incubator (25 +/- 1°C). Mortality was assessed daily. After death, the insects were placed in a humid chamber (15 × 60 mm Petri dish, lined with filter paper and moistened weekly with 150µl of sterile water) in an incubator (25 +/- 1°C) to promote fungal sporulation. This was done to check if the larvae *causa mortis* was the inoculated fungus. Only larvae that presented signs of fungal sporulation were included in the analysis.

Our aim here was to select one isolate of each fungal species with similar virulence to perform the next experiments, giving equal chances to each fungus in the infection process *a priori*. One isolate each of *Beauveria bassiana* (B0265) and *Metarhizium anisopliae* (S2107) were selected to perform all subsequent coinfection experiments as they had the same LT50 at 1×10^6 spores ml^{-1} (see Results). For simplicity, we will refer to these as *Beauveria* and *Metarhizium* throughout the remainder of the text.

2.4 Coinfection with parasites inoculated at the same time and in the same place

In this experiment, we investigated the outcome of coinfections in which the parasites were inoculated together at the same time and in the same place on the insect's body. For this, 1 ml of each single fungal suspension was prepared at 1×10^6 spores ml^{-1} as described in 2.2, representing the single inoculation treatment. The mixed fungal suspension was prepared by combining 500µl of *Beauveria* and 500µl *Metarhizium* suspensions, both at 1×10^6 spores ml^{-1}, resulting in a final suspension of 1ml (1:1). One microliter (approximately 1000 spores) of single and mixed fungal suspensions were then inoculated in 25 larvae (n=25) in the following treatments: 1 – only *Beauveria*; 2 – only *Metarhizium*; 3 – *Beauveria* + *Metarhizium*; 4 –

Control (blank 0.01% Tween 80). The inoculation process and the conditions of this experiment were the same as those described in 2.3. Host mortality was assessed daily. After death, the insects were placed in individualized humid chambers

After twenty-five days in a humid chamber, the insect cadavers were inspected under a stereomicroscope (Olympus SZ61) to record sporulation by each fungal species or by both given the percentage of fungal sporulation. To release the spores and create a suspension, each cadaver was placed in an Eppendorf tube with 1.5 ml of 0.01% Tween 80 solution and vortexed for 1 minute, followed by 10 minutes in an ultrasonicator. They were then kept in the refrigerator at 5°C for 24 hours. The tubes were then centrifuged at 14,000 rpm for 10 min at room temperature, vortexed for another minute to resuspend and homogenize the suspensions. Spore production was estimated for each species in a Neubauer Improved Chamber under a microscope (Nikon Eclipse E200). *Beauveria* and *Metarhizium* have different spore morphologies, and they are easily distinguished under a microscope (Supplementary Figure 2).

2.5 Location of infection

To assess the importance of spatial proximity of infection sites between parasites in determining the outcome of coinfection, we inoculated the fungi in the same or in different locations on the host's body. Single and mixed suspensions of *Metarhizium* and *Beauveria* (1×10^6 spores ml^{-1}) were prepared for this experiment as described in 2.4. When the fungi were inoculated separately, 0.5µl of each single fungal suspension was applied, totalling 1µl. When they were inoculated together, 1µl of a mixed suspension was applied per individual. The selected locations on the insect's body were the second thoracic segment ("front"; as above) and the antepenultimate abdominal segment ("rear"), as they are the most distant segments of the insect's body in which it is possible to perform efficient inoculation, as determined in prior tests. Therefore, the experiment consisted of 5 treatments (n=25): 1 – Coinfection in the front (coinfection-blank); 2 – Coinfection in the rear (blank-coinfection); 3 – *Beauveria* front + *Metarhizium* rear (B-M); 4 – *Metarhizium* front + *Beauveria* rear (M-B); 5 – Control. Time taken to kill the host, the percentage of fungal sporulation and spore production were evaluated as above.

2.6 Order of arrival and time between infections

To test whether the order of arrival and time between infections can change the outcome of the coinfections, we inoculated *Beauveria* and *Metarhizium* simultaneously or spaced at intervals of 12 hours. The experimental treatments were as follow: 1 – simultaneous inoculation; 2 – first inoculation at the beginning of the experiment and the second 12 hours later; 3 – first inoculation at the beginning of the experiment and the second 24 hours later; 4 – first inoculation at the beginning of the experiment and the second 36 hours later; 5 – first inoculation at the beginning of the experiment and the second 48 hours later; 6 - Control. We inoculated 0.5µl of each fungus at 1×10^{6} spores ml^{-1} in the insect's front (see above), totaling 1µl per individual. Two experiments were carried out in the same conditions: (i) *Beauveria* first then *Metarhizium;* and (ii) *Metarhizium* first then *Beauveria*. The time taken to kill the host and the percentage of fungal sporulation were evaluated.

3. Statistical Analyses

The analyses were conducted using the software R (version 4.2.1).

Host mortality: Survival data were used to obtain survival curves using Kaplan–Meier estimators and Median Lethal Times (LT50). Curves were compared using χ^2 Log-Rank tests and pairwise comparisons between treatments were carried out with Bonferroni corrections ($p < 0.05$). Only larvae that presented signs of fungal sporulation were included in the analyses.

Percentage fungal sporulation: Percentage data were analyzed using a generalized linear model (GLM) assuming a quasi-binomial distribution. Simplification was done by amalgamating levels within categorical variables one by one from the full model. Models were compared using F-tests ($p< 0.05$).

Number of spores: Numbers of spores were compared between treatments using a GLM assuming a Quasi-Poisson distribution. Model simplification by backward stepwise deletion was carried out so as to inspect consequent changes in deviance. Considering that the treatments where we coinfected the larvae had three possible outcomes – only *Metarhizium* sporulated, only *Beauveria* sporulated or both sporulated – the number of number of repetitions (n) for each outcome might be low. In those situations, we carried out a Permutational analysis of variance (PERMANOVA) with 1,000 interactions, taking 10 samples per treatment in each interaction. Thus, the influence of our treatments on the number of spores was confirmed using *adonis2* function in the vegan R package (Oksanen et al., 2018).

4. RESULTS

4.1 Virulence bioassay for isolate choice

The three screened isolates were pathogenic to *T. molitor* and decreased the survival probability over time (*Beauveria* B0265 –- $\chi^2_{(4)}$=115, p<0.0001; *Metarhizium* M0122 – $\chi^2_{(4)}$=130, p<0.0001; *Metarhizium* S2107 – $\chi^2_{(4)}$=125, p<0.0001; Supplementary Figure 1). The insects' exposure to the three isolates resulted in 100% mortality at the concentrations of 10^6 and 10^7spores ml^{-1}. The median survival time for 10^6 spores ml^{-1} differed between the isolates, at 5 days for *Beauveria* B0265 and *Metarhizium* S2107 and 6 days for *Metarhizium* M0122. The pairwise comparison of survival between B0265 and S2107 at 10^6 spores ml^{-1} was not statistically significant (p=1.0). We chose therefore to perform all subsequent experiments with *Beauveria* B0265 and *Metarhizium* S2107 at 10^6 spores ml^{-1}, since both isolates have a similar profile of speed to kill at this concentration.

4.2 Coinfection with parasites inoculated at the same time and in the same place

The first coinfection experiment was conducted to obtain baseline data. Here, the survival rate of *T. molitor* differed among treatments ($\chi^2_{(4)}$=79.7, p<0.0001). However, this difference was only observed between the infection treatments and the control (*Beauveria* vs. control p<0.0001; *Metarhizium* vs. control p<0.0001; coinfection vs. control p<0.0001). The median survival time was 5 days in all infection treatments and pairwise comparison between single and mixed inoculations did not differ statistically (coinfection vs. *Beauveria (*p=1) and coinfection vs. *Metarhizium* (p=0.079); Figure 1A). The virulence of coinfections is therefore the same as single infections by each fungus.

As expected, single infections resulted in 100% of the cadavers with the emergence and sporulation of the respective inoculated fungus. In the coinfection treatment, 60% of dead larvae showed only signs of *Beauveria*, 12% only of *Metarhizium* and 28% of both fungi (Figure. 1B).

When the two fungi were successfully established in coinfections, as observed by both fungi sporulating (28% of replicates; Figure. 1C), *Beauveria* produced only 16% of the spores ($4.0\times10^7 \pm 1\times10^7$ spores ml^{-1} – we give means ± SE throughout) that it produced in the single infections ($2.4\times10^8 \pm 2\times10^7$ spores ml^{-1}; $F_{1,29}$=25.731, p<0.0001)**.** However, when only *Beauveria* sporulated (60% of replicates), it produced a similar quantity of spores (2.2×10^8 ±

3.2×10^{7} spores ml^{-1}) to the quantity it produced in the single infections ($F_{1,38}$=0.5018, p=0.482).

A similar pattern was found with *Metarhizium* and the Permanova analysis (due to low n – see above) showed that the number of spores produced by *Metarhizium* differed between single and coinfections (R^2=0.238, p=0.012). In the coinfections, *Metarhizium* produced only 28% of the spores ($7.2\times10^{7} \pm 3.2\times10^{7}$ spores ml^{-1}) that it produced in the single infections ($2.6\times10^{8} \pm 1\times10^{8}$ spores ml^{-1}) ($F_{1,29}$=13.653, p<0.0009; Figure. 1C). As with *Beauveria* above, when only *Metarhizium* sporulated (12% of replicates), it produced a similar quantity of spores ($2.6\times10^{8} \pm 3.2\times10^{7}$ spores ml^{-1}) to the quantity it produced in the single infections ($F_{1,30}$=0.002, p=0.958).

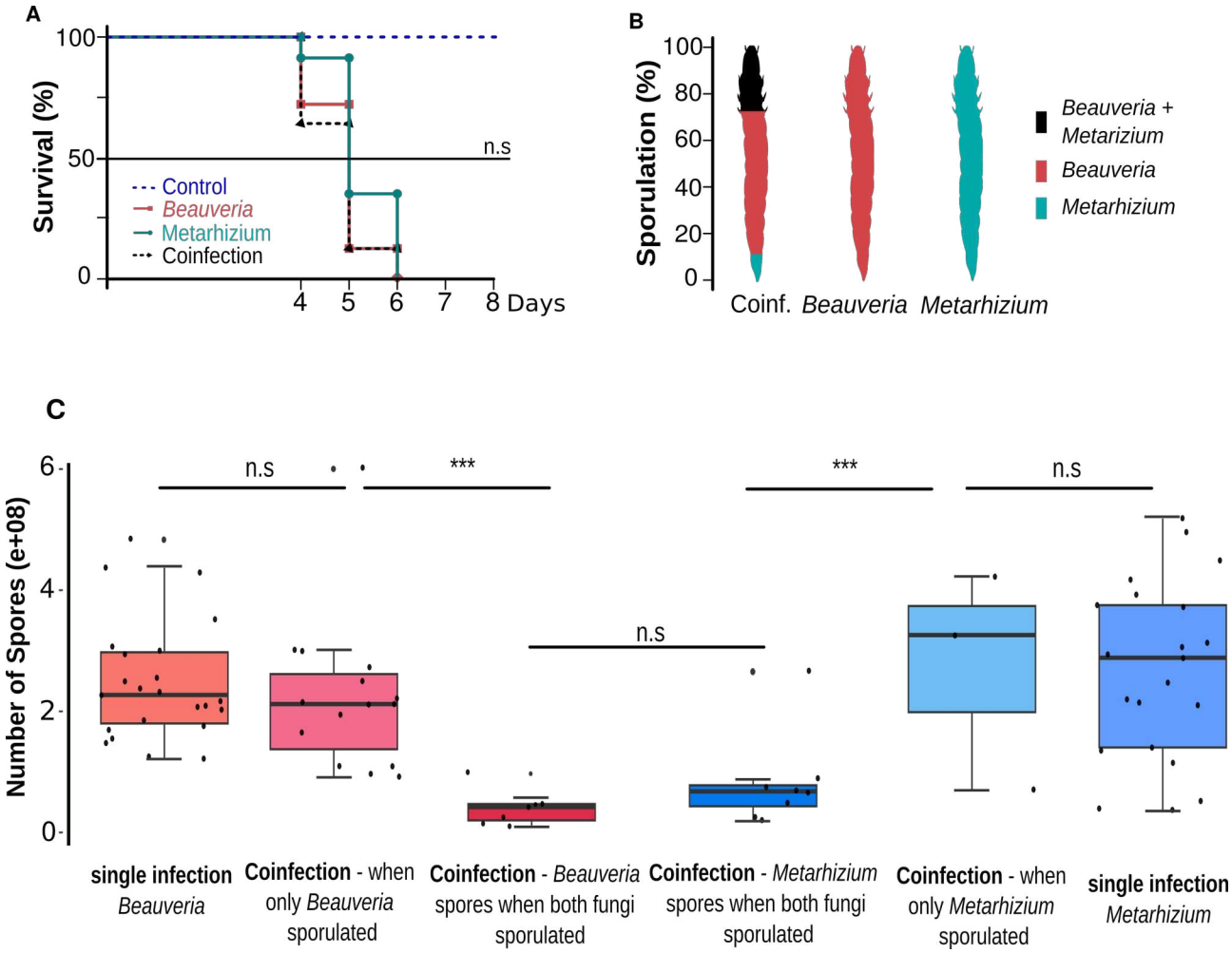

Figure 1. Cumulative survival, percentage of fungal sporulation and fungal spore production in *Tenebrio molitor* larvae with single and mixed infections of *Beauveria bassiana* and *Metarhizium anisopliae*. (A) Cumulative survival of *T. molitor* in single infection and coinfection. The insects (n=25) were topically

inoculated on the second thoracic segment with 1µl of single or mixed suspensions (1×10^6 spores ml^{-1}). Larval mortality was assessed daily during 10 days. (B) Percentages of dead larvae in which a single fungal species or both sporulated after 25 days in humid chamber. (C) Numbers of spores produced by *B. bassiana* and *M. anisopliae* in single infections and coinfections after 25 days in a humid chamber. *p≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

4.3 Location of infection

In this experiment, we examined how the outcome of coinfections might be altered when the parasites infect the host in different locations of the body *versus* the same location. Coinfection in the same and different locations of the host's body affected the survival probability over time compared to controls ($\chi^2_{(4)}$=79.7, p<0.0001; Figure. 2A), but comparisons of survival between infection treatments were not significant (p=1.0 for all pairwise tests).

The percentages of coinfected cadavers of *T. molitor* differed between our 4 treatments ($F_{3,85}$=3.028, p=0.033; Figure. 2B). When the parasites were inoculated in the same location, the percentages of coinfected individuals did not differ ($F_{1,86}$=0.946, p=0.333) and were 19.0% and 30.0% (coinfection-blank and blank-coinfection, respectively). However, this percentage doubled ($F_{2,87}$=7.64, p=0.006) when they were inoculated in different locations (45.8% and 58.3% (B-M and M-B, respectively).

Given that the number of individuals that had signs of both fungi was higher when they were inoculated in distant parts of the host, we also evaluated if this had an impact on the number of spores produced by the parasites in this situation. Permanova analysis showed that the number of spores produced when both fungi sporulated differed between the treatments (R^2=0.291, p=0.008). When *Beauveria* was inoculated in the front and *Metarhizium* in the rear, the number of spores produced in coinfection (B-M – $1.6\times10^8 \pm 1.8\times10^7$ spores ml^{-1}; Figure. 2C) was higher than the other three treatments ($F_{1,31}$=10.59, p= 0.003). In contrast, there was no greater production of spores when *Metarhizium* was inoculated in the front and *Beauveria* in the rear than the treatments with inoculation in the same location (M-B – $9.6\times10^7 \pm 1.3\times10^7$ spores ml^{-1}; $F_{1,32}$=1.825, p= 0.186). Meanwhile, there was no difference in the number of spores produced in coinfection when both fungi were inoculated together in the rear part *versus* the front part (blank-coinfection – $7.8\times10^7 \pm 1.1\times10^7$ spores ml^{-1}; coinfection-blank – $6.9\times10^7 \pm 1.1\times10^7$ spores ml^{-1}; $F_{1,31}$=0.139, p=0.711).

We also measured the number of spores produced by *Beauveria* when this was the only fungus to sporulate and there were no differences between treatments($F_{1,36}$=0.794, p= 0.505; Figure. 2D). We did not assess this for *Metarhizium* alone sporulating due to the low number of replicates in all treatments.

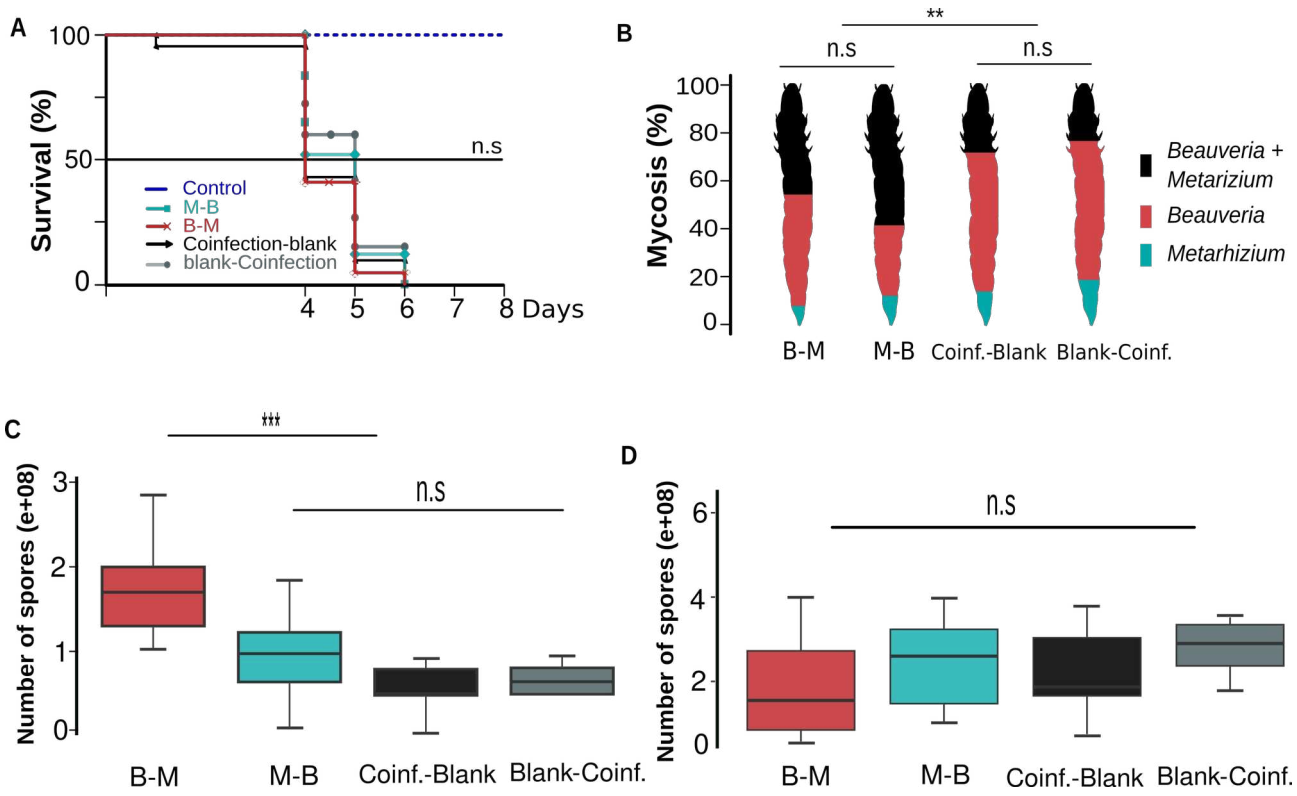

Figure 2. Cumulative survival, percentage of fungal sporulation of *Tenebrio molitor* larvae and number of spores produced in coinfections between *Beauveria bassiana* and *Metarhizium anisopliae* in different location of inoculation (see Location of infection experiment description for details). A) Cumulative survival of *T. molitor* in single and coinfection in different locations. The insects (n=25 per treatment) were topically inoculated with 1µl of mixed suspensions (1×10^6 spores ml^{-1}). Larval mortality was assessed daily for 10 days. (B) Percentage of dead larvae with sporulation by a single fungal species or by both after 25 days in humid chamber. (C) Number of spores produced when *B. bassiana* and *M. anisopliae* sporulated in the coinfection treaments after 25 days in humid chamber. (D) Number of spores produced when only *B. bassiana* sporulated in the coinfection treatments after 25 days in humid chamber . *p≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

4.4 Order of arrival and time between infections

Here we investigated how the order in which the two parasites infect the host, and also the time elapsed between these infection events, might affect the outcome of coinfections. Two experiments were conducted, one with *Beauveria* first, the other with *Metarhizium* first. When *Beauveria* was the first inoculated fungus, the larvae started to survive longer than simultaneous infection after 24 hours between the first and second inoculation (0h vs 24h, $p<0.0001$; 0h vs 36h, $p=0.008$; and 0h vs 48h, $p=0.0007$). When *Metarizhium* was inoculated first, the time span between infections did not alter *T. molitor* survival compared to simultaneous inoculation (0h vs 12h, $p=0.12$; 0h vs 24h, $p=1.00$; 0h vs 36h, p=027; and 0h vs 48h, $p=1$; Figure. 3C).

The first parasite to arrive completely dominated the infection process and the percentage of fungal sporulation. *Beauveria* colonized 45% of insects when inoculated together with *Metarhizium*. This increased to 95% when *Metarhizium* was inoculated 12h later, to 90% with a 24h gap, and to 100% with 36h and 48h gaps ($F_{2,98}=28.676$, $p<0.0001$ for simultaneous versus all asynchronous inoculations; $F_{1,98}=0.379$, $p=0.539$ among the three asynhchronous inoculation treatments; Figure. 3B).

The same pattern was observed when *Metarhizium* was the first fungus to arrive, the % of colonized corpses by *Metarhizium* was different between simultaneous and sequential infections ($F_{2,109}=38.271$, $p<0.0001$; Figure. 3D), but did not differ between the time lapse of infections ($F_{1,109}=0.064$, $p=0.799$). *Metarhizium* colonized 22.7% in simultaneous infection, 68% when it was inoculated 12h first, 95% in 24h first, and ~95% in 36h and 48h first.

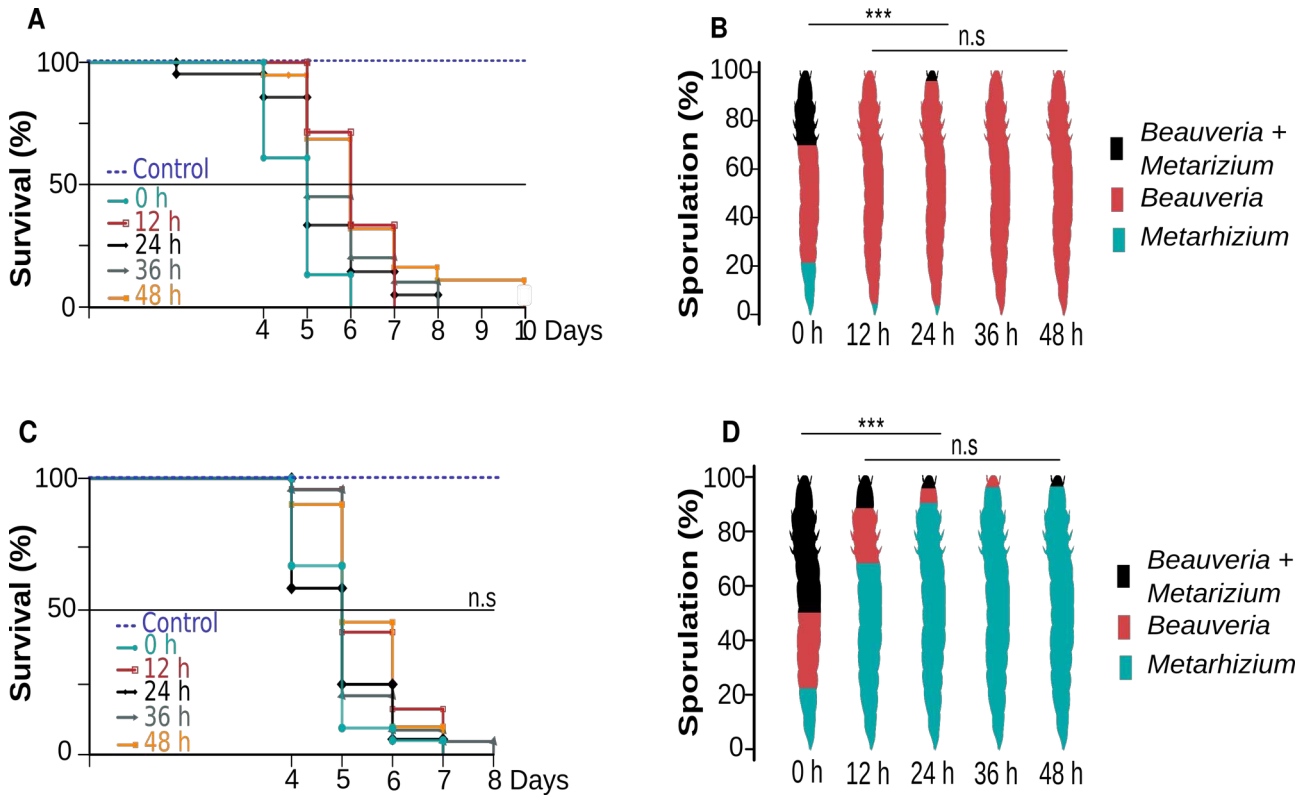

Figure 3. Cumulative survival and percentage of fungal sporulation of *Tenebrio molitor* larvae in coinfections between *Beauveria bassiana* and *Metarhizium anisopliae* in different order of inoculation and time between infections (see. Order of arrival and time between infections for details). (A,C) Cumulative survival of *T. molitor* in simultaneous and sequential inoculations. (A) *B. bassiana* was the first inoculated fungus followed by *M. anisopliae.* (C) *M. anisopliae* was the first inoculated fungus followed for *B. bassiana.* The insects (n=25 per treatment) were topically inoculated on the second thoracic segment with 1µl of mixed suspensions (1×10^6 spores ml^{-1}). In total, 1µl of spores suspension (1×10^6 spores ml^{-1}) was applied in each larva, 0.5µl for each fungal species. Larval mortality was assessed daily for 10 days. (B,D) Percentage of fungal sporulation by a single fungal species or by both after 25 days in humid chamber – B: only *Beauveria*; M- only *Metarhizium*; B+M- both fungi. *p≤0.05; **p ≤ 0.01; ***p ≤ 0.001.

5. Discussion

Our aim in this study was to set up a study system to investigate coinfections using similar parasites, where the outcome of coinfection between them would not be strongly related to their contrasting identities. *Beauveria* and *Metarhizium* represent good model organisms for this purpose due to their relatedness and shared characteristics as facultative parasites with similar infection mechanisms, host exploitation strategies and transmission modes. We first screened *Beauveria bassiana* and *Metarhizium anisopliae* isolates to select one of each with similar virulences towards *T. molitor* larvae. The selected isolates performed similarly at a concentration of 1×10^6 spores ml^{-1} in a mortality assay, both presenting an LT50 of 5 days (see 4.1 and fig. S1).

In the first coinfection experiment, the two fungi were inoculated together at the same time and place, which is the simplest and most common scenario in coinfection studies when the parasite mode of infection is the same. There was no effect of coinfection on virulence towards the host, measured here as survival time (Fig. 1A). It is common for studies of coinfections to consider effects on virulence, especially when there are practical concerns such as the possibility to use mixtures in biological pest control with potential additive, synergistic or interference effects on virulence. This has received particular treatment in a recent meta-analysis by (Tinsley et al. *in prep*) and is not the focus of our study. We are using a relatively high dose to infect the host – in 6 days all insects died in single infections and in coinfections – and this can make it difficult to detect differences in virulence. It is worth noting, however, that any study of coinfections is faced with the choice of whether to use full or half doses of each parasite and there is not necessarily any correct choice among the two. Here we chose to use half doses of each parasite to give the same total dose as the other treatments. This would, we expect, lead to similar levels of activation of host immune defences to the single infections with which we are comparing our coinfections. Our choice of half doses making up the same total dose as in individual infections should limit the variation in immune defence, in line with this experimental approach.

On the parasite side, even though the virulences of the two isolates established a *priori* were identical, the outcome of coinfection differed for the two. When we inoculated the two fungi together, 60% of the cadavers appeared to have been colonized only by *Beauveria,* 12% only by *Metarhizium,* and 28% by both species (Fig. 1B). For simplicity, we will assume that cadavers only presenting one of the fungi emerging and sporulating were colonized only by

that fungus as the ‘winner’ of that interaction, but of course we would expect there to have been some growth of the ‘loser’ before the other fungus dominated the resource (this is the subject of future study). These results show clear evidence of interspecifc competition between the two fungi and *Metarhizium* suffered more negative impacts than *Beauveria.* Our study was conducted at a fixed temperature of 25±1°C and we know from previous work that temperature can strongly affect the performance of these fungi within live hosts (Mishra et al., 2015). We also know that there is variation between isolates in their performance across temperatures (e.g. Garcia et al., 2016). We expect therefore that there is a temperature at which we would find the two isolates to perform equally well and we are indeed undertaking this work.

Over a quarter of the cadavers had both fungi sporulation. This is much higher than two previous studies in which Li et al. (2021) and Pauli et al. (2018) found that virtually all insects submitted to coinfection of *Beauveria* and *Metarhizium* at the same time presented signs of sporulation by only one of the two fungi. Their results support a pattern of strong competitive exclusion between entomopathogenic fungi. In both studies, however, the virulences of the tested isolates were different, giving a clear advantage to the more virulent strain to dominate the infection process. Here we took care to use strains with similar virulences, which likely resulted in a considerable percentage of cadavers producing both fungi. Competitive exclusion was also the predominant pattern in coinfection, since only one fungus dominated almost three-quarters of the cadavers, however, we suggest that original virulence of each isolate might play an important role in which dominant species will emerge.

The percentage of fungal sporulation by each fungus provide valuable information on the relative performance of each isolate in coinfections. However, this could be misleading when it comes to evaluating the fitness of each fungus. To address this, we measured the production of spores as an additional measure. This revealed a pronounced effect of coinfection in reducing the number of spores (Fig. 1C; Supplementary Figure 2). This only occurred when both fungi were able to sporulate. However, in coinfections where one fungus did manage to dominate the cadaver, its spore production was not significantly lower than that in single infections.

We have not yet examined the mechanism by which each parasite interacted with the other. We can, however, characterize it as interference competition. We also suggest that the parasite that manages somehow to suppress its competitor – to the point where the competitor

is not able to emerge from the cadaver and sporulate – is able to use the resources available in the cadaver to the full extent. Where the parasite does not manage to suppress its competitor to this degree, and the competitor is still able to emerge and sporulate, both parasites suffer extreme reductions in fitness. This type of competition probably occurs through the production of toxic metabolites, which are known to be a fungal defensive strategy (Künzler, 2018). In fact, entomopathogenic fungi produce a range of secondary metabolites that could act as antimicrobial agents (Zhang et al., 2020). Direct competition for host resources also limits parasite replication, as demonstrated in mixed viral infection of lepidopterans (Ishii et al. 2002), while competition through activation of the host's immune defences can have a similar effect (Balmer and Tanner, 2011). In both of these scenarios, however, we would expect far less suppression of the less dominant parasite than we find here, leading us to suspect the first pattern (via toxins) to be of prime importance here.

In the second coinfection experiment, we examined how spatial separation between the sites of infection might affect the outcomes of coinfection and our hypothesis is that when the host is infected in distant parts of its body, this will enhance the chances of successfully establishment of both parasites. Theoretical models in community ecology predict that a spatial pattern of intraspecific aggregation combined with interspecific segregation will promote species coexistence (Pacala and Levin 1998). This is indeed the pattern when we examine the percentages of cadavers in which both fungi were able to sporulate. This increased from ~19% when fungi were inoculated in the same location to 58% when they were inoculated in different parts of the body (Fig. 2B). We might *a priori* expect a scenario in which parasite is able to establish infection to some degree before suffering the consequences of competition with the other. This would be expected more with direct fungus-fungus interactions, via local production of toxins or competition for host resources, than with interactions via host defences, as we would expect the latter to be stimulated systemically throughout the host's body. Different infection locations could delay competition between both parasites, increasing the time and availability of resources for successful establishment of both species. Entomopathogenic fungi need to attach and penetrate the insect cuticle to infect their hosts, and competition can begin at this stage. Li et al. (2018) found that *Metarhizium robertsii* inhibits spore germination of *Beauveria bassiana*, diminishing the chances of infection by the latter. The physical space between the infections in our experiment may have eliminated this early negative interaction, enhancing the chances of both species to succeed

and produce more spores. Once inside the hosts, these fungi also may compete directly through interference competition or indirectly by resource competition (Mideo, 2009). Interference may be enhanced when both parasites are closely located at the beginning of the infection (Pedersen and Fenton, 2007), although it is possible that parasites could interact remotely via toxins released into the haemolymph. Parasite invasion in different parts of the host will decrease this proximity so may lead to a milder form of early competition. Also, parasites start to use host resources available in the vicinity and the physical distance of a competing organism could impact the efficiency of early resource acquisition. Thus, different locations of infection enhanced the chances of coexistence in our model system and consequently the fitness of both parasites.

When both fungi emerged from the host in this second experiment, the number of spores was higher only when *Beauveria* was inoculated in the front and *Metarhizium* in the rear (Fig. 2C). This was expected since we believe that competition was alleviated in this situation where the parasites were inoculated in distant parts of the host. However, this was not the case with *Metarhizium* in the front and *Beauveria* in the rear. The number of spores in this situation did not differ from when both parasites were inoculated in the same place. Albeit surprising, this might have occurred due to local interaction between the fungi and the insect immune system. Changing the location of infection between the parasites enhanced the chances of coexistence and the number of spores produced in when both fungi sporulated in coinfection, however when competitive exclusion took place and only one fungus was able to produce spores, the presence of the other competitor did not impact the ultimate fitness of the "winner". This can be seen in the situation when only *Beauveria* emerged in coinfection treatments, where the number of spores did not change (Fig. 2D).

In the third coinfection experiment, inoculations were spaced by time intervals of up to 48 hours. This was the only situation in which we found an effect of the treatments on host survival, specifically when *Beauveria* was inoculated first (Fig. 3A). It might be possible that *Beauveria* adopts the inhibition strategy plastically when a competitor arrives later on the scene. Since defensive metabolites can be costly (Shwab and Keller, 2008), the divergence of resources from virulence factors and/or resource acquisition to defence might explain the decrease in virulence in this scenario. Our hypothesis for this experiment is that the first parasite to arrive will have an advantage colonizing the host and that this advantage will be gradually enhanced the later the second parasite enters in the system. Priority effects in

colonizing habitats usually indicate an advantage for the first to arrive (Kennedy et al., 2009; Sarneel et al., 2016) and the expectation here is that the first fungus would be able to establish itself before encountering the second. This was indeed the pattern observed: regardless of species, the first fungus to arrive dominated the infection and outcompeted the second (Fig. 3B and 3D). This result endorses the current evidence that priority effects appear to be a widespread phenomena in a biological context (Stroud et al., 2024) and that the first parasite to arrive will be dominant (Karvonen et al., 2019 for a review), but see Clay et al. (2018). In simultaneous inoculation, *Metarhizium* proved to be a weak competitor compared to *Beauveria.* However, this weak competitor was able to outcompete its rival when it was inoculated first (within-host priority effect). The first fungus to arrive may generate a hostile environment for the second, which could enable the establishment of the first. For example, inside their hosts, entomopathogenic fungi will multiply as hyphal bodies or blastospores to facilitate the consumption of host resources. Moreover, insect's cellular and innate immune response will be activated followed by the first parasite invasion (Hillyer, 2016). Thus, the physical presence of the first parasite, its secondary metabolites and the activation of host immune response may act together to inhibit the subsequent establishment of another fungus.

We did not detect any effect of time lapsed between the first and second fungi inoculation in parasite success, contradicting the second part of our hypothesis. In our experiment, 12 hours represents our first experimental interval between the first and the second inoculation, and this time between infections was sufficient to assure the dominance of the first inoculated parasite. This is evidence of a strong within-host priority effect in our study system, even for *Metarhizium.* Zilio and Koella (2020) found an asymmetrical effect of timing between infections depending on which microbe arrived first. However, in this study, the transmission strategies of the parasites were different (horizontal vs. vertical) and this may have influenced the outcome. The effects of timing between infections in virulence or parasite fitness have been investigated considering different host ages in parasite infection (Izhar, et al. 2015; Bem-ami, 2019). The short period of time between the first and second infection in our experiment did not represent a clear difference in host age, thus we believe that our results rely only on a prior dominance of the first fungus. It would be interesting to shorten the time between infections (less than 12 hours) to determine when the priority effect begins and if this can be related to some within-host process.

Our results support the adoption of this system to study the dynamics of coinfections. We were able to select variables related to the process of infection and investigate its effects on coinfection. The physical proximity of the infection sites, for example, is a neglected aspect in this research area and we demonstrate here that it can have an impact on the outcome of coinfections. This knowledge can be used to understand the dynamics of coinfection, and also to construct a body of theory that could be considered in other biological systems. We intend to build on the findings of this model system testing other environmental variables, such as host nutrition and temperature. Since our findings are based on the infection by two obligate killer parasites with similar host resource acquisitions and transmission modes, we are not able to test the effects of niche occupation inside the host in our system, for example. Thus, the interpretation of our results must take into account this characteristics. Different methodological approaches can address other questions, for example Lello et al. (2018) developed a study using taxonomy, immune response, and characteristics of the parasitological niche to successfully predict the outcome of coinfection across species boundaries.

Finally, *Beauveria* spp. and *Metarhizium spp.* are the most used fungi in biological control of insects in the world, and the mixture of these agents has been considered to overcome some inconsistencies in control (Inglis et al., 1997). Based on our results, the virulence towards *T. molitor* was not enhanced in most of our coinfections experimental treatments. Also, when both fungi were established, the number of spores produced by each one was diminished, representing a fitness reduction for these potential biocontrol agents. Depending on the control strategy adopted, say where some secondary cycling may increase or prolong a biopesticide’s efficiency, this could represent a loss in efficiency. However, more environmental factors must be tested to check if fungi coinfections could enhance virulence in changing conditions and of course, these findings would need to be evaluated in other insect systems with the strains of fungi of interest in a given biocontrol programme.

6. SUPPLEMENTARY MATERIAL

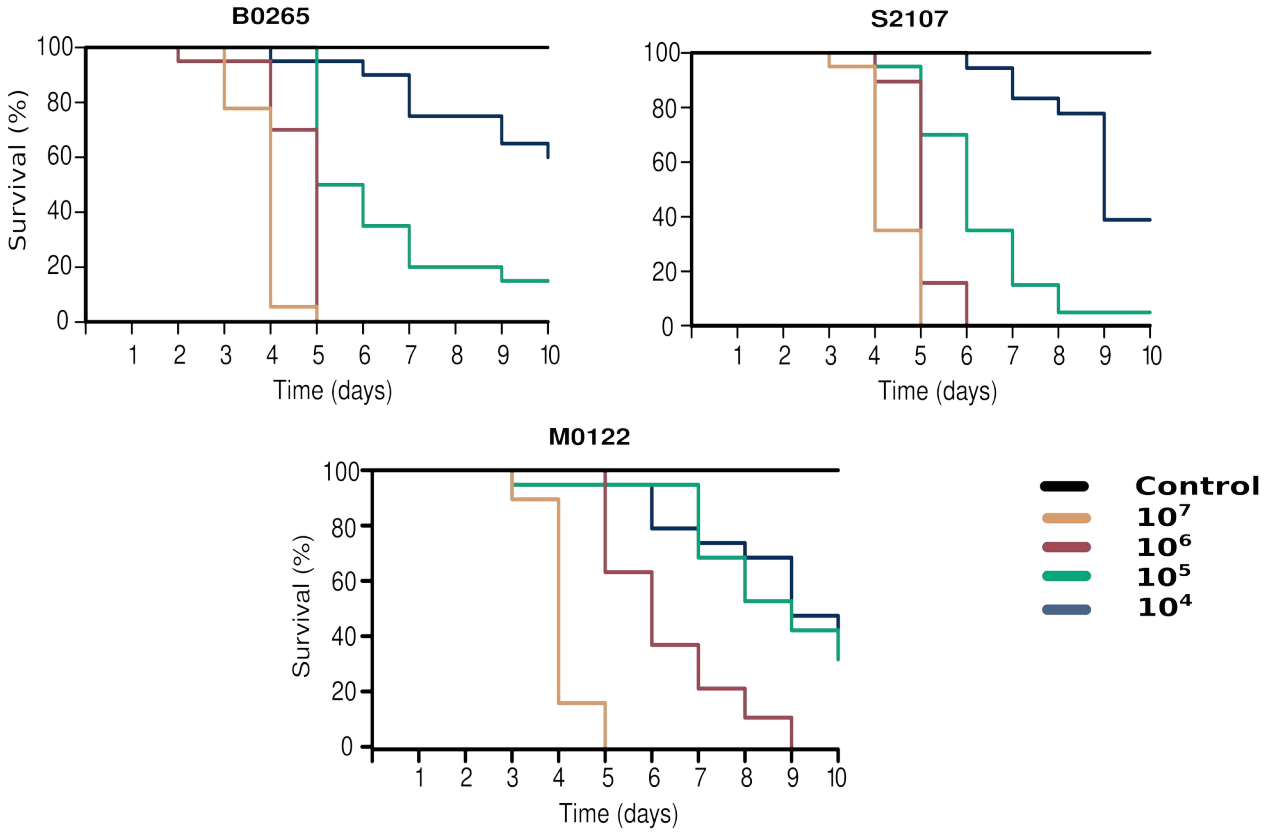

Figure S1 - Cumulative survival of *Tenebrio molitor* larvae exposed to one isolate of *Beauveria bassiana* (B0265) or one of two isolates of *Metarhizium anisopliae* (S2107 and M0122) at four different doses (1×10^4, 1×10^5, 1×10^6 and 1×10^7 spores ml^{-1}). The insects (n=20 per treatment) were topically inoculated on the second thoracic segment with 1µl of each isolate × dose combination. Larval mortality was assessed daily during 10 days.

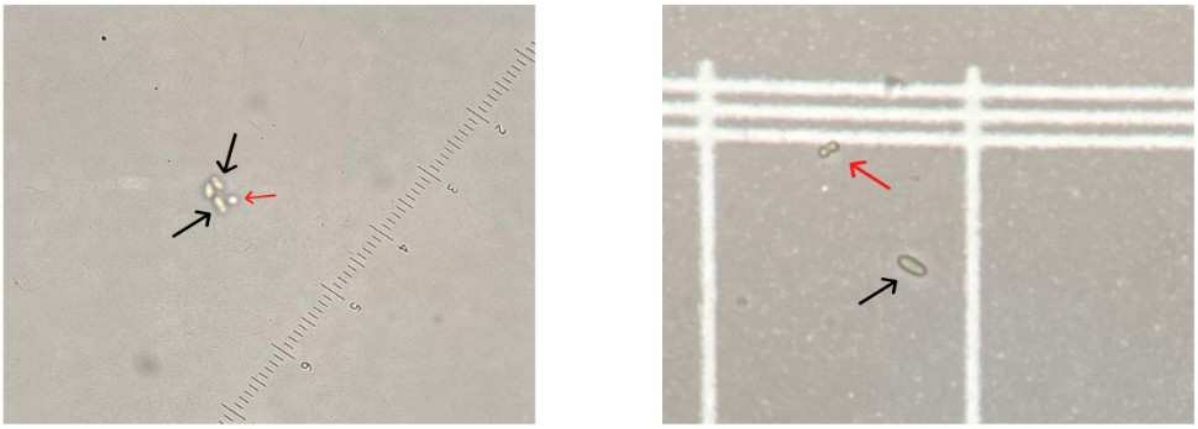

Figure S2 – Morphological discrimination of spores of *Beauveria bassiana* and *Metarhizium anisopliae* under a light microscope (40×). *Beauveria* spores are indicated with red arrows and presented the typical morphology of this species, with a globose to subglobose structure. *Metarhizium* spores are indicated with the black arrows and also presented its typical morphology with cylindrical spores.

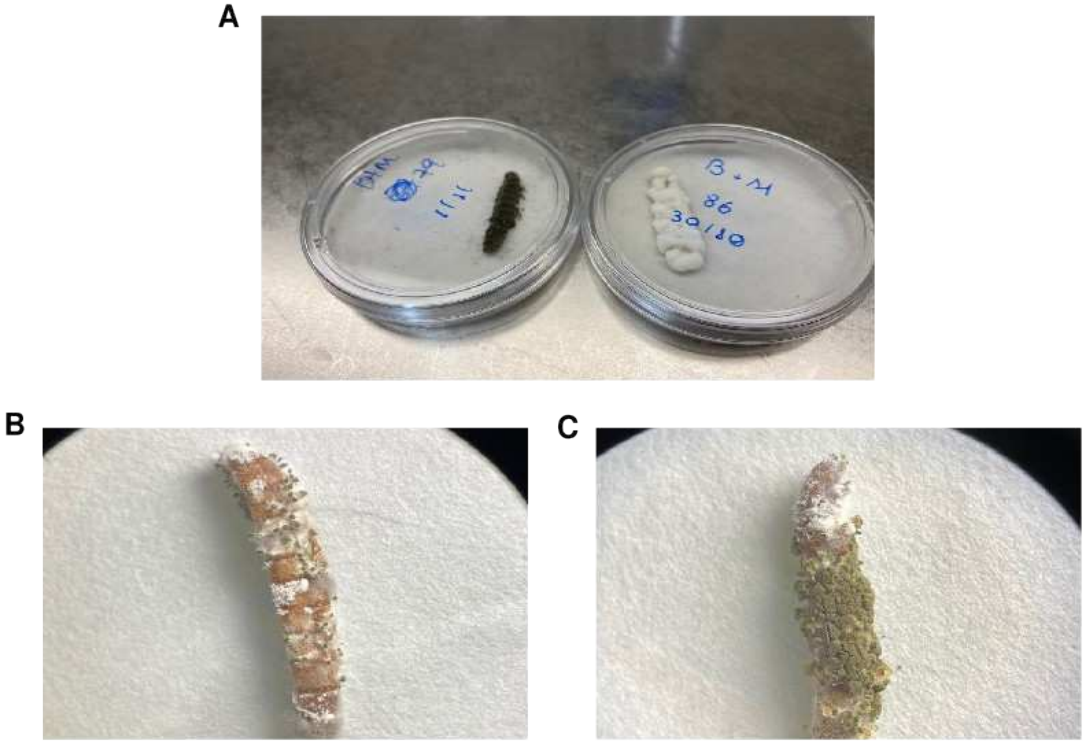

Figure S3 - Larvae of *Tenebrio molitor* showing signs of sporulation by *Beauveria bassiana* and *Metarhizium anisopliae*. **(A)** Larvae showing signs of sporulation by only one fungal species, *Metarhizium* in the left Petri dish, *Beauveria* in the right dish. **(B-C)** Co-inoculated larvae showing signs of sporulation by both fungi species.

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CHAPTER II

Nutrition and fungal infections: macronutrients influence host mortality but have no effect on coinfection and parasite fitness

Nutrition and fungal infections: macronutrients influence host mortality but have no effect on coinfection and parasite fitness

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ABSTRACT

Host nutrition is a major modulator of host-parasite interactions. The composition of food ingested by hosts could impact the outcome of diseases and this has been demonstrated especially in bacterial and viral diseases. However, the effect of host nutrition on fungal infections remains largely unexplored. Using a model system with the beetle *Tenebrio molitor* as a host and two entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* as parasites, we investigated the effect of macronutrients - protein and carbohydrate - on host and parasite fitness in fungal single and coinfections. We inoculated *T. molitor* larvae with single and coinfection of *Beauveria* and *Metarhizium* and assigned them to three chemically-defined diets varying their Protein: Carbohydrate (P: C) ratio from 1:5, 5:1 or 1:1. Our results show that the effect of nutrition on coinfection was limited. Host mortality was higher on larvae fed on a carbohydrate biased diet (1:5) than the other treatments. This means that larvae submitted to the three infection treatments performed poorly on the 1:5 diet. On the parasite side, *M. anisopliae* produced more spores after host death in all three diets compared to *Beauveria* or coinfection. Notably, the total amount of food eaten was lower on the carbohydrate diet compared to the proteinacious and balanced diets, irrespective of type of infection. Our results contradict prior evidence suggesting that a diet rich in carbohydrate could help insects to fight fungal diseases. We also demonstrated that regardless of host nutrition, *M. anisopliae* is more efficient in spore production after host death and this could represent a fitness advantage for this genus in our study. Together, our results emphasize the complexity of nutritional effects on host health and parasite fitness and highlights the need of more studies with different host and parasite taxa to build robust patterns in this field.

1. INTRODUCTION

Host nutrition has received significant attention in recent years as a key modulator of host-parasite interactions (Ponton et al., 2023). Numerous studies have shown that hosts experiencing malnutrition could be more susceptible to parasite infection and/or suffer more from disease once infected than well nourished hosts (Katona and Katona-apte., 2008; Kangassalo et al., 2015; McKay et al., 2016). For instance, mortality was increased by 50% in starved bumblebees after a chronic infection by the gut parasite *Crithidia bombi* (Brown et al., 2000). Similarly, diet restriction, but not starvation, also demonstrated similar negative effects on host resistance to infections in *Drosophila sp.* (Ayres and Schneider, 2009; Meshrif et al., 2022). In both scenarios, these outcomes may have occurred due to the host immune system being compromised under conditions of nutritional stress (Moret and Schmid-Hempel, 2000; McKay et al., 2016).

Additional studies have indicated that it is not just the quantity of food that matters, but also the balance of macronutrients such as protein (P) or carbohydrate (C) ingested (eg. Deans et al., 2022). The role of macronutrients in parasitism has become an important topic within the field of nutritional ecology (Simpson and Raubenheimer, 2012; Ponton et al., 2013). Currently, there is mixed evidence regarding the effects of both of these macronutrients (P and C) on disease from the host's perspective, depending on the study system. For example, caterpillars of *Spodoptera litorallis* and *Spodoptera exempta* were were more resistant to a virus and a bacterial infection (respectively) when fed a diet with a higher protein-to-carbohydrate (P:C) ratio (Lee et al., 2006; Povey et al., 2009). The beneficial effects of protein can be attributed to a positive effect on the host immune response (Wilson et al., 2019). On the other hand, a carbohydrate-biased diet can be better for fighting infections in the Australian plague locust *Chortoicetes terminifera* (Graham et al., 2014; Srygley 2023) and in *Drosophila melanogaster* (Ponton et al., 2019). Graham et al. (2014) suggest that higher mortality of locusts fed a protein-rich diet when infected with the fungal parasite *Metarhizium acridum*, could be due to a greater efficiency of the fungus in exploiting protein available in the host's body than the host itself. However, the mechanisms behind these effects are still poorly understood and these mixed results highlight the need of more studies to identify similarities across host and parasite taxa (Cotter and Al Shareefi., 2021). Fungal infections, for example, are considerably less explored in this context than viral or bacterial infections.

Further, our understanding of the effect of host nutrition on disease has focused on one host – one parasite interactions. Nevertheless, a single host can be infected by multiple parasites concurrently, leading to a coinfection scenario (Petney and Andrews, 1998; Cox, 2001). The within-host interaction between parasites is one of the main determinants of outcomes of coinfections (Pedersen and Fenton, 2007), and they can interact directly or indirectly. Indirect interaction between parasites can occur through the host immune system or competition for shared resources and in both cases host nutrition can play a sigfnificant role (Ezenwa, 2021). For example, in the mouse *Mus musculus,* infection with the microparasite *Mycobacterium bovis* had a positive effect on the nematode *Nippostrongylus brasiliensis* nematode by weakening the host immune response, but only when hosts were protein limited (Budischak, 2015). In insects, studies that have investigated the effect of host diet on coinfections have focused exclusively on food quantity (Fellous & Koella, 2010; Reyserhove et al., 2017; Zilio & Koella, 2020, Deschodt and Cory, 2022) so there is little or no information on the effect of diet quality on the results of coinfections.

Based on the model system established in the first chapter using *Tenebrio molitor* and *Beauveria bassiana* and *Metarhizium anisopliae*, we investigated the effect of diet quality on fungal single and coinfection. The particularities of our model system are relevant here to investigate the effects of nutrition on fungal infections. Both fungi are obligate killers (*sensu* Ebert and Weisser 1997) with similar mechanisms of infection and host exploitation (see Hajek and St. Leger, 1994), and belong to the same taxonomic order (Hypocreales). These features will allow us to focus experimentally on dietary factors that can impact fungal infections and limit the influence of basic biological aspects of disparate organisms on the outcome.

We used three chemically defined diets varying in their protein and carbohydrate content (%Protein:%Carbohydrate: 35:7, 21:21 and 7:35) to access the effect of diet quality on the outcome of single and coinfections for the host and for the parasite. We choose 35:7 and 7:35 P:C ratios to represents extreme proportions of both macronutrients on host diet. On the host side, the consequences of parasite challenge was estimated through survival rate after infection. On the parasite side, we evaluated the number of spores produced after infection when the host was fed on these diets. In addition, protein, carbohydrate, and total food comsumption were recorded to check any indication of an increase or reduction in ingestion of specific nutrients following a fungal infection.

2. MATERIAL AND METHODS

2.1 Insect colony

Larvae of *Tenebrio molitor* L. were obtained from Livefoods® - United Kingdom - and reared in open plastic trays (21×17×9 cm) in an incubator at 25±1°C, LD 12:12 photocycle, and 65 ± 10% relative humidity. Larvae were supplied *ad. libitum* with a diet composed of a mix of oat bran, wheat fiber, and wheat bran (1:1:1). Five slices of carrot were provided every 4 days. Trays with larvae were checked twice a week and individuals which had pupated were manually transferred to new plastic trays and covered with a sheet of paper to allow emerged adults to hide. After adult emergence, they were transferred and maintained in plastic containers (41×26×7 cm) with the same diet plus carrot slices. Once a month, newly emerged larvae and eggs were collected.

2.2 Fungal suspensions

The fungi isolates *Beauveria bassiana* B0265 and *Metarhizium anisopliae* S2107 were used in all experiments (Chapter 1). Fungal suspensions were prepared by flooding fungal cultures cultivated on PDA (Potato Dextrose Agar) plates with 10ml of 0.01% Tween 80 and scraping the conidia with a Drigalski spatula. The suspensions were then stirred for 1 min. and filtered through sterile gauze. This procedure allowed the separation of the conidia from hyphal fragments. Conidial concentrations were estimated in a Neubauer Improved Chamber and adjusted to the desired concentration through serial dilutions. The suspensions were stored at 5°C and conidial viabilities were determined one day before the experiment. For this, an aliquot of 100 µl of the suspension was individually plated on BDA culture medium and incubated at 25°C for 18 h. Suspensions were only used if they reached 95% germination. Stock concentrations were estimated using a Neubauer Improved Chamber and ten-fold serial dilutions were prepared and adjusted to the dose 1×10^{7} conidia ml^{-1}

2.3 Artificial diets

Three experimental diets varying in their soluble protein and digestible carbohydrate content were used in this study (%Protein:%Carbohydrate: 35:7, 21:21 and 7:35) following the protocol of Simpson and Abisgold, 1985. Protein and carbohydrate portions made up 42% of the final diet and other constituents were Wesson's salts (2·4%), cholesterol (0·5%), linoleic

acid (0·5%), ascorbic acid (0·3%) and a vitamin mixture (0·2%). The remaining portion was made up of cellulose (54%). Dry ingredients were suspended at a 1–6 ratio w/v in 1% agar solution and were presented to the larvae in a wet form. We choose 35:7 and 7:35 P:C ratios to represents extreme ratios of both macronutrients to investigate its effects on single and coinfections outcomes.

2.4 Infection protocol

One ml of each of the *Beauveria* and *Metarhizium* suspensions was prepared at 1×10^{7} conidia ml^{-1} as described in 2.2, representing the single inoculation treatments. The mixed fungal suspension was prepared by combining 500µl of *Beauveria* and 500µl *Metarhizium* suspensions, both at 1×10^{7} conidia/ml, resulting in a final suspension of 1ml (1:1). One microliter (approximately 1,000 spores) of single or mixed fungal suspensions were then topically inoculated on to the second thoracic segment of *T. molitor* larvae and each insect was monitored until the liquid component of the suspension completely penetrated its cuticle (chapter 1). If the suspension came into contact with the polypots or the filter paper, the larva was replaced as we felt the total dose may have been reduced in these cases. The control group was inoculated only with 1µl of Tween 80.

2.5 Experimental design

On the day of the experiment, 72 larvae were weighed individually on a microbalance and placed in 25 ml polypots. The larvae were then randomly split into four infection groups: only *Beauveria* (n=18), only *Metarhizium* (n=18), *Beauveria+Metarhizium (*n=18), Control (n=18). The infection protocol was described in 2.3. After fungal inoculation, each infection group was divided into three different diet treatments, P:C 1:5, 1:1, 5:1 (n= 6 per treatment). Larvae were kept at 25±1°C and 65 ± 10% relative humidity in an incubator. Diet blocks were provided daily to the larvae and consumption was estimated as the difference between the initial and final dry weight of each diet block. The uneaten and the control diets were dried to a constant mass for approximately 24 hours allowing the consumption per larva to be estimated (Lee et al., 2006). The experiment was repeated 3 times in the same conditions.

2.6 Survival, % mycosis and spore counting

Host mortality was assessed daily. After death, the insects were placed in individualized humid chambers (25mL pots lined with filter paper and moistened weekly with 100µl of distilled water) in an incubator (25 +/- 1°C) to stimulate fungal sporulation. After twenty-five days in a humid chamber, the insect cadavers were carefully inspected under a stereomicroscope and we recorded how many insect corpses showed signs of sporulation by each fungal species or by both given the percentage of mycosis. Also, when we estimated spore production in a Neubauer chamber in a microscope we could confirm which fungus had sporulated. The corpses were placed in an Eppendorf tube with 1.5 ml of 0.01% sterile Tween 80 solution and vortexed for 1 minute. They were then placed in an ultrasonicator for 10 minutes and kept in the refrigerator at 5°C for 24 hours. After 24 hours, the tubes were vortexed for another minute until the suspensions became homogeneous. Spore production was estimated in a Neubauer improved chamber.

2.7 Statistical analysis

Survival curves were fitted through Kaplan-Meier analysis to estimate host survival curves and Median Lethal Times (LT50). A Cox proportional hazard (PH) model was used to determine the effect of diet and infection on survival time (Sheng and Gosh, 2020). Model simplification by backward stepwise deletion was carried out to amalgamate levels. Numbers of spores were compared between treatments using a GLM assuming a Quasi-Poisson distribution. Model simplification by backward stepwise deletion was carried out so as to inspect consequent changes in deviance (Crawley, 2015). To analyse cumulative intakes of protein and carbohydrate, we fitted generalized linear models (GLM) to test for the effects of infection treatment and diet. Tukey *post hoc* tests were used to determine differences in mean total cumulative protein and carbohydrate intakes between treatments.

3. RESULTS

3.1 Protein, carbohydrate and total consumption

Protein and carbohydrate daily intakes were measured for larvae restricted to P:C 1:1, 1:5 and 5:1 for 12 days. However, we chose to compare the total consumption during the initial 5 days when the first larvae began to die to fungal infection.

Only the P:C ratio ($F_{3,138}$=1854 P < 0.001) had an effect on protein intake, the infection treatment ($F_{2,141}$=2.646, P=0.0515) and the interaction between the two factors ($F_{6,132}$=1.755, P=0.1131) were not significant (Fig. 1). As expected, larvae restricted to the protein biased diet consumed more protein then the other two (we give means +/- se on Table 1). Post hoc tests showed that comparision between all diet grous were highly significant 1:1 – 1:5 p<0001; 1:1 – 5:1 p<0001; 1:5 – 5:1 p<0001 (Fig. 1).

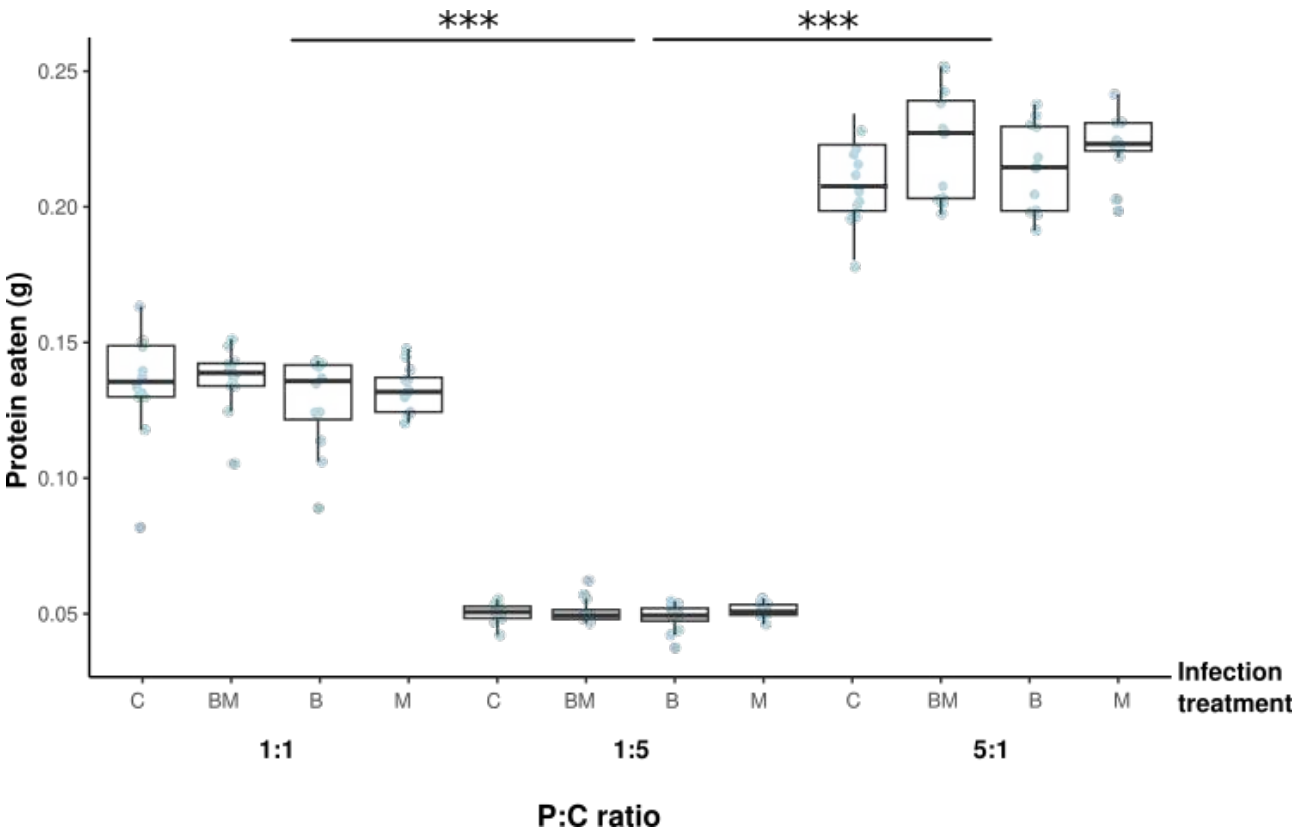

Figure 1. Protein intake of *Tenebrio* molitor larvae restricted on three different diets. The protein intake was estimated daily for non infected larvae (control) and larvae infected with *Beauveria, Metarhizium* or both. Diet blocks of diet were provided daily to the larvae and consumption was estimated as the difference between the initial and final dry weight of each diet block. Box-and-whisker plots show median and interquartile range (IQR)**.** Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001). The

infection treatment groups were represented in each diet as follow: C – control group; BM- coinfection; B – *Beaveria bassiana;* C- *Metarhizium anisopliae.*

Similar to protein intake, only diet ($F_{2,138}$=1498, P < 0.001) had an effect on carbohydrate intake, the infection treatment ($F_{3,134}$= 1.337, P=0.264) and the interaction ($F_{6,132}$=0.648, P=0.691) was not significant (Fig. 2). As expected, larvae restricted to the carbohydrate-biased diet consumed more carbohydrate then the ones restricted to the other two diets (we give means +/- se on Table 1). *Post hoc* tests showed that comparision between all diet groups were highly significant 1:1 – 1:5 p<0001; 1:1 – 5:1 p<0001; 1:5 – 5:1 p<0001.

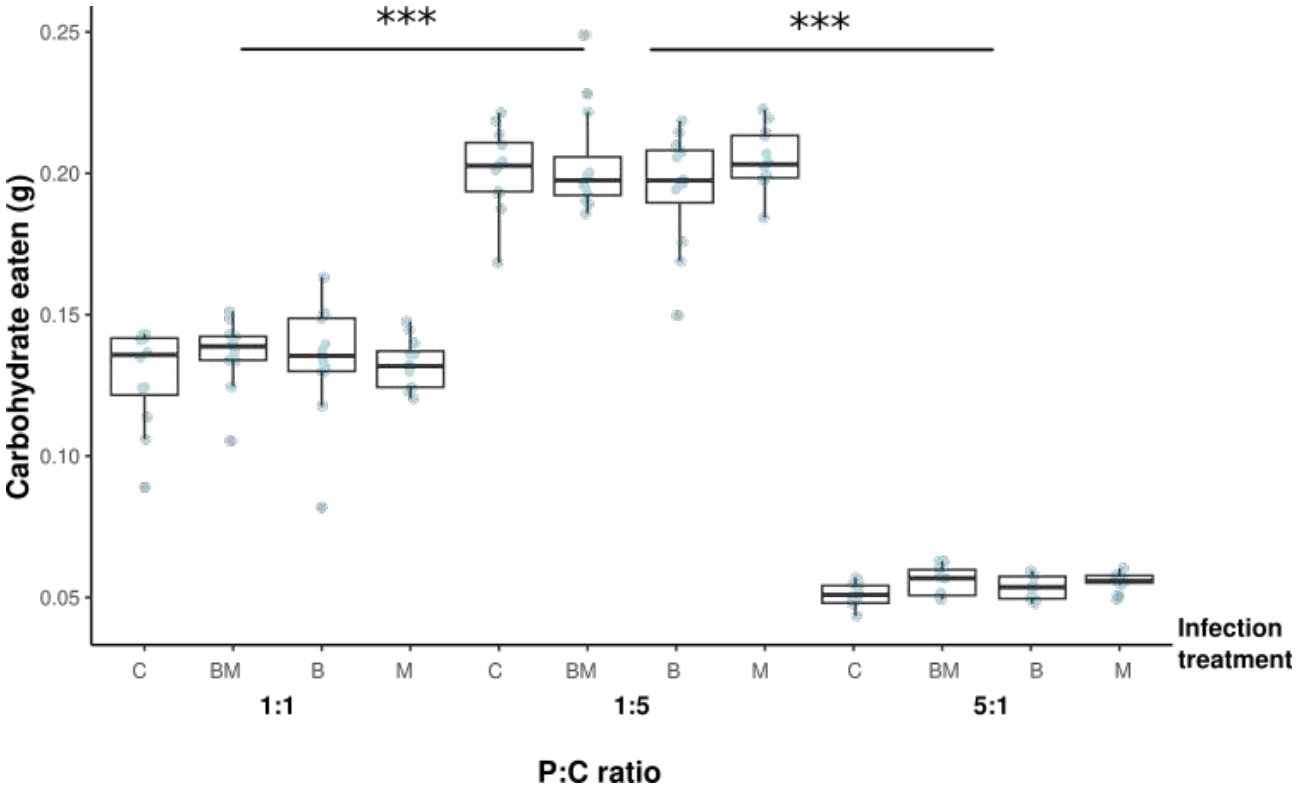

Figure 2. Carbohydrate intake of *Tenebrio* molitor larvae restricted on three different diets. The carbohydrate intake was estimated daily for non infected larvae (control) and larvae infected with *Beauveria, Metarhizium* or both. Diet blocks of diet were provided daily to the larvae and consumption was estimated as the difference between the initial and final dry weight of each diet block. Box-and-whisker plots show median and interquartile range (IQR)**.** Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001). The infection treatment groups were represented in each diet as follow: C – control group; BM-coinfection; B – *Beaveria bassiana;* C- *Metarhizium anisopliae.*

Regarding the total of food eaten by the end of the fifth day, only the P:C ratio ($F_{2,138}$=7.580, P =0.0007) had an effect on total intake, the infection treatment ($F_{3,140}$= 2.299, P=0.0801) and the interaction ($F_{6,132}$=0.877, P=0.5131) were not significant (Fig.5). Post hoc tests showed that larvae restricted to the protein biased diet (5:1) and a balanced diet (1:1) ate more than larvae on a carbohydrate diet (1:5) (1:1 – 1:5 p= 0.0134; 1:1 – 5:1 p=0.670; 1:5 – 5:1 p=0.0008 (Fig. 3).

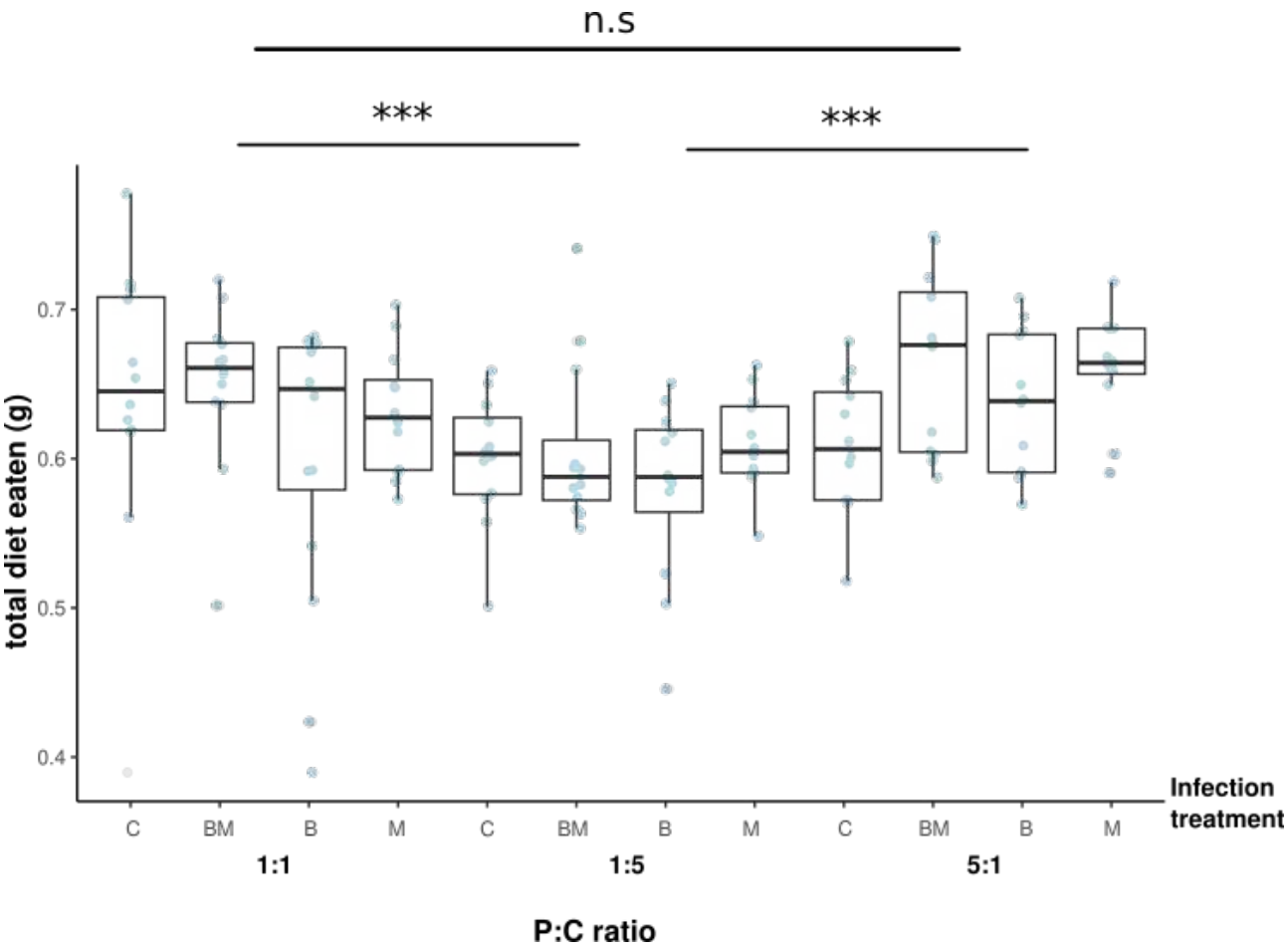

Figure 3. Total diet consumption of *Tenebrio* molitor larvae restricted on three different diets. Total consumption was estimated daily for non infected larvae (control) and larvae infected with *Beauveria, Metarhizium* or both. Diet blocks were provided daily to the larvae and consumption was estimated as the difference between the initial and final dry weight of each diet block. Box-and-whisker plots show median and interquartile range**.** Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001). The infection treatment groups were represented in each diet as follow: C – control group; BM-coinfection; B – *Beaveria bassiana;* C- *Metarhizium anisopliae.*

Table 1. Consumption [g ± standard error (SE)] of non infected (control) or infected *Tenebrio molitor* with *Beauveria*, *Metarhizium* or both in three chemically defined diets after five days.

Diet (P:C ratio)	Infection treatment	Consumption (g) mean ± se		
		Protein	**Carbohydrate**	**Total eaten**
1:1	Control	0.1344 ± 0.0059	0.1344 ± 0.0059	0.6403 ± 0.0282
	Beauveria	0.1282 ± 0.0050	0.1282 ± 0.0050	0.6109 ± 0.0240
	Metarhizium	01324 ± 0.0087	01324 ± 0.0087	0.6307 ± 0.0120
	Beauveria + *Metarhizium*	0.1363 ± 0.0034	0.1363 ± 0.0034	0.6493 ± 0.0164
5:1	Control	0.2045 ± 0.0044	0.0511 ± 0.0011	0.6086 ± 0.0174
	Beauveria	0.2140 ± 0.0046	0.0535 ± 0.0011	0.6369 ± 0.0125
	Metarhizium	0.2223 ± 0.0034	0.0559 ± 0.0008	0.6618 ± 0.0093
	Beauveria + *Metarhizium*	0.2232 ± 0.0058	0.0558 ± 0.0014	0.6643 ± 0.0163
1:5	Control	0.0486 ± 0.0014	0.1946± 0.0058	0.5793 ± 0.0133
	Beauveria	0.0503 ± 0.0010	0.2014± 0.0042	0.5994 ± 0.0137
	Metarhizium	0.0512 ± 0.0007	0.2050 ± 0.0031	0.6102 ± 0.0102
	Beauveria + *Metarhizium*	0.0509 ± 0.0013	0.2039 ± 0.0054	0.6069 ± 0.0172

3.2 Effect of host diet on survival after fungal single and coinfection

Diet affected host survival in infected insects($\chi^2_{[2]}$= 7.239, p=0.0267) (Fig. 4). However, the type of fungal infection (single with *Beauveria* or *Metarhizium* and coinfection) did not influence survival ($\chi^2_{[2]}$= 5.315, p=0.070) nor the interaction between diet and infection ($\chi^2_{[4]}$= 3.011, p=0.555). Larvae on a carbohydrate biased diet (1:5) survived less then the other treatments ($\chi^2_{[2]}$= 6.546, p=0.021) with 55.5%, 77.7% and 72.22% of deaths at day 12 after infection by *Beauveria*, *Metarhizium* and coinfection, respectively. Larvae on a 1:1 and 5:1 diets did not present more than 50% of deaths irrespective of fungal inoculation. The experiment was performed three times and the effect of run on the results was not significant $\chi^2_{[5]}$= 5.735, p=0.332.

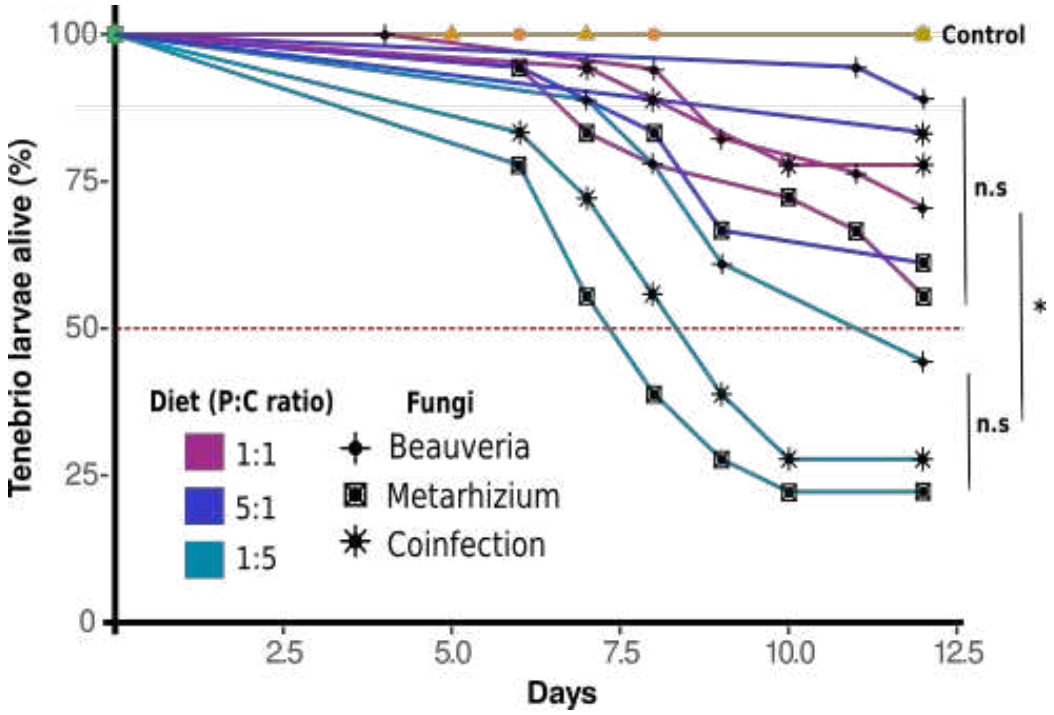

Figure 4. Cumulative survival *Tenebrio molitor* larvae exposed to single and mixed infections of *Beauveria* and *Metarhizium* in different diets. (A) Cumulative survival of *T. molitor* in single and coinfection. The insects (n=18) were topically inoculated with 1µl of single and mixed suspensions (1×10^7 spores ml^{-1}). Diets were replaced daily and larvae mortality was assessed daily for 12 days.

3.2 Effect of host diet on number of spores produced by parasites

Fungal identity had an impact on the number of spores produced by the parasites at the end of the infection ($F_{2,61}$=6.169, p=0.003) (Fig. 5). Host diet ($F_{2,63}$=1.251, p=0.293) and the interaction between diet and fungus infection ($F_{4,57}$=0.167, p=0.953) were not significant. The number of spores produced by *Metarhizium* was approximately three times higher ($7.1\times10^7 \pm 2.8\times10^7$ spores ml^{-1} – we give means ± SE throughout) then *Beauveria* ($2.7\times10^7 \pm 0.886\times10^7$ spores ml^{-1}) and than coinfection ($2.97\times10^7 \pm 1.10\times10^7$ spores ml^{-1}).

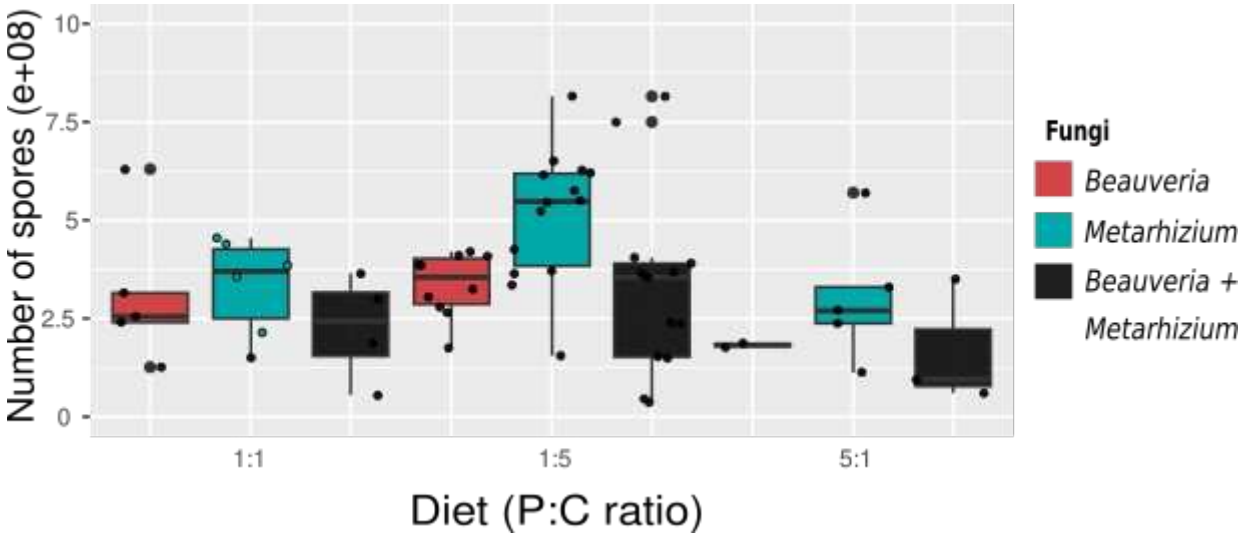

Figure 5. Number of spores produced after an infection in single and coinfections between *Beauveria* and *Metarhizium* in three chemically defined diet P:C ratio 1:1, 1:5 and 5:1. Hosts were placed in a humid chamber after death for 25 days to stimulate fungal sporulation. The corpses were placed in an Eppendorf tube with 1.5 ml of 0.01% sterile Tween 80 solution and vortexed for 1 minute. Spore production was estimated in a Neubauer improved chamber. Tukey *post hoc* tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001).

4. DISCUSSION

Using an entomopathogenic fungus model system, we demonstrated the role of macronutrients – protein and carbohydrate – on host survival, parasite spore production, and diet consumption in an insect host. Nutrition can impact the course of a disease and considering its impact on both sides of the host-parasite relationship provides us a broader perspective on the effects of nutrition on infection outcomes.

The consumption of protein, carbohydrate and total diet amount was estimated until day 5, when the first insects began to succumb to the fungal infection. As expected, the insects restricted to the protein-biased diet had higher intakes of protein by the end of the fifth day, while those on the carbohydrate-diet consumed more carbohydrate. The type of fungal infection did not alter the consumption pattern of protein and carbohydrate. *Beauveria* and *Metarhizium* are hypocrealean fungi with similar host exploiration strategy and nutrient acquisition methods (Hajek and St. Leger, 1994), potentially explaining the analogous response observed and why hosts did not change the feeding behaviour in single or coinfection between these fungi. Interestingly, the total amount of food eaten was lower on the carbohydrate diet compared to the proteinaceous and balanced diets, irrespective of type of infection. *Tenebrio molitor* larvae can be demanding on protein (see below), but it might be possible that the costs of eating more carbohydrate to achieve the ideal protein amount are high and a total reduction of food is prioritized in this situation.

Coinfection did not change the pattern of single infections on host mortality across the three diets. Hosts restricted to a carbohydrate-biased (1:5) diet survived less compared to the other diets following all infection treatments. In this diet, the larvae consumed the smallest amount of protein between treatments and this may have negatively affected immune function and escalated to higher mortality in this group. Protein is a major resource to immune function in insects and a diet with a higher P:C ratio can enhance immune parameters in immature stages of lepidopteran caterpillars, including encapsulation response, antimicrobial activity, and phenoloxidase production (PO) (Lee et al. 2008; Brunner et al. 2014, Wilson et al., 2018). The upregulation of these immune parameters enhances resistance against invading pathogens, resulting in higher survival rates following a pathogen challenge (Povey et al., 2013). However, studies using fungal parasites often find that a carbohydrate diet is better to combat infection. In orthopterans, this has been shown at least twice. The locust *Chortoicetes terminifera* had higher survival rates (45%) after *Metarhizium* inoculation on a high-

carbohydrate diet compared to the high-protein diet (25%) (Graham et al., 2014). In the grasshopper *Melanoplus sanguinipes,* the *Metarhizium*-inoculated individuals survived longer eating the carbohydrate rich diet and the protein rich diet. This means that the insects performed better in both extreme diets compared to the balanced one (21P:21C) (Zembrzuski et al., 2023). The authors suggest that a higher protein intake can boost the insect immune system, helping them against parasites, while the carbohydrate rich diet may starve the pathogen of protein, slowing parasite replication.

Our results are in contrast with these findings, but it is worth noting that locusts and grasshoppers are hemimetabolous insects while *Tenebrio molitor* is holometabolous, undergoing distinct phases (egg, larval, pupal and adult phase). This can dramatically influence the nutrition acquisition and demands at each stage (Chapman et al., 2013). Protein is often recognized as a key nutrient during the larval phase of holometabolous insects (Nash and Chapman, 2014), and this also might be the case after a parasite challenge since there is substantial evidence of protein as an important modulator of larval immunity (Lee et al., 2006; Povey et al., 2009, Wilson et al., 2019). In this context, it is possible that the effect of macronutrients on T. molitor larvae after a fungal infection is more similar to the response of caterpillars infected by a virus or bacterium than to grasshoppers infected by fungi.

We estimated parasite fitness based on the number of spores released after infection. Host diet did not affect the number of spores produced by *Beauveria* and *Metarhizium* in single or coinfection scenarios. Deschodt and Cory (2022) also found no impact of diet quantity on parasite fitness when *Trichoplusia ni* larvae were coinfected with *B. bassiana* and Baculovirus. The authors suggest that the production of transmission stages might be primarily related to cadaver size and the effects on parasite fitness will be only noticeable when food quantity is sufficiently low to impact the development of the host. We weighed larvae individually at the beginning of the experiment, and weight did not impact mortality. Therefore, we do not expect abrupt changes on larval weight at the time of death, and this might have resulted in no differences in spore production between diets.

However, *Metarhizium* consistently produced more spores than *Beauveria* or coinfection, reagardless of diet treatment. Once inside the host, entomopathogenic fungi grow as hyphal bodies or blastospores, colonizing the host haemocel. This growth and proliferation utilize the pool of host resources available in the hemolymph and can be affected by the availability of carbon and nitrogen. Some studies have shown that the carbon-to-nitrogen C/N

relationship on culture medium can impact the growth and spore production of entomopathogenic fungi. For example, Ali et al. (2009) found that all isolates of *Isaria fumosoroseus* exhibited maximum colony growth rate in a high C/N medium in vitro. Safavi et al., 2007 found high variation in the optimal C/N ratio between the three isolates of *B. bassiana* and one of *M. anisopliae*, which means that the optimal ratio can vary greatly between isolates tested. Additionally, spore production also can be influenced by the C/N ratio *in vitro* and this also seems to depend on the isolate. A medium with a low C/N ratio can be a better substrate for spore production in some isolates of *B. bassiana* and *M. anisopliae*, for example (Wyss et al., 2001; Kamp & Bidochka, 2002). This pattern of higher spore production in a low C/N medium was confirmed by Vega et al., 2003, but this appears to be to be genus-specific. The specificity of nutrient requirements for each fungal genus/isolate may explain why *Metarhizium* performed better across all diets. Further, experiments checking the nutritional requirements of our isolates could corroborate the spore production results in this study.

Our results involving the infection of a holometabolus insect larva by two fungal parasites and the coinfection between them adds more host and parasite taxa information into the nutritional ecology field. Host mortality was higher on a carbohydrate biased diet irrespective of fungal treatment and this does not match the previous findings of fungal infections in hemimetabolous insect. The effect of host nutrition on infection might vary greatly between host and parasite taxa and our findings corroborate this pattern, highlighting the relevance of adding new species combination in these studies. On the parasite side, the impact of host diet was not observed on parasite fitness and this could be because of species or isolate specificities. Thus, the nutrient demands of each isolate might be different and the profile of each isolate could be necessary to detect any changes in pathogen sporulation. Since *Beauveria* and *Metarhizium* are widely used in insect biological control programs, it could also be interesting to check how the effect of host nutrition can take place using hosts plants. Crop plants might differ in their P:C ratios (Bernays and Chapman, 1994; Schoonhoven et al., 2005), even different tissues in the same plant (Deans et al., 2016). In this sense, the efficiency of these fungi can be influenced by crop plants in a way that could decrease or enhance insect mortality.

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CHAPTER III

Temperature modifies the outcome of coinfection in an insect-parasitic fungus system

Temperature modifies the outcome of coinfection in an insect-parasitic fungus system

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Keywords: insect-fungi interactions, host-parasite interactions, *Metarhizium*, *Beauveria*

ABSTRACT

Temperature and coinfections are seen as modulators of host-parasite interactions. However, they are usually investigated in isolation and empirical evidence of the effect of temperature on infection by two or more organisms is lacking. Temperature could impact coinfections through changes in key life-history traits of hosts and parasites. These changes can give rise to a myriad of infection outcomes with variable consequences for them. Using the model system proposed in this thesis, we investigated the effect of temperature on the outcome of coinfection between *Beauveria bassiana* and *Metarhizium anisopliae* on the beetle *Tenebrio molitor*. We performed two *in vitro* experiments – germination and growth – to evaluate the patterns of interaction between both fungi at four different temperatures: 18, 22, 26 and 30°C. Afterwards, we coinfected *T. molitor* larvae with *Beauveria* and *Metarhizium* and assigned them to the same temperatures. Our results show that *Beauveria* had higher germination rates in isolation, but there was no clear pattern of inhibition of germination of one fungus on the other. Also, *Beauveria* presented greater growth at 26°C and *Metarhizium* at 30°C. This partially matches our *in vivo* experiment where the dominance of *Beauveria* colonizing the infection process was diminished at 30°C. From the host's perspective, mortality was higher at 26°C and this might be caused by the metabolic peak of entomopathogenic fungi at this temperature. Changes in the interaction between hosts and parasites or parasites and parasites are expected in a climate change scenario with higher temperatures being common in human-modified and natural habitats. Our results bring together *in vitro* and *in vivo* experiments to shed light on the effects of temperature in an insect-parasitic fungus system. We showed that a rise in temperature brings changes in coinfection outcomes with consequences for the hosts and parasites involved.

1. INTRODUCTION

The environment surrounding hosts and parasites is variable, and many biotic and abiotic factors can influence their relationship. Temperature is one of those factors with major roles on host and parasite functional traits (Paull et al., 2015; Iltis et al., 2019). In ectothermic hosts, for example, several physiological and behavioral processes are up-regulated in warmer temperatures, but only up to a certain threshold, beyond which the continuous rise in temperature will decrease host performance (Kingsolver, 1993; Angilletta 2009). Parasite traits can also be modified by temperature (Thomas and Blanford, 2003); for example mycelial growth and spore production in entomopathogenic fungi (Seib et al., 2022; Ekesi et al., 1999) and replication in some viruses (Kobayashi et al., 1981) can be affected. Ectothermic hosts and their parasites serve as valuable biological systems to investigate the effect of temperature on disease dynamics and species interactions, because their performances are strongly dependent on environmental temperature for their biological and physiological processes (Dell et al., 2011).

The influence of temperature on host and parasite traits can change the outcome of an infection. The water flea *Daphnia dentifera* reared at warmer temperatures had more robust physical barriers to prevent infection, but decreased cellular immune responses (Sun et al., 2023). When *D. dentifera* were infected by a fungal parasite *Metschnikowia bicuspidata* and reared at warmer temperatures, they experienced reductions in fecundity and lifespan compared to those reared at milder temperatures. On the parasite side, Ragonese et al. (2023) observed that higher temperatures (34°C) led to a decrease in parasite replication rate within the host. This effect was not attributed to enhanced host immunity or accelerated host development, but rather reflected the thermal limits of the parasite itself. Ultimately, the temperature-mediated changes in these traits could potentially impact infection outcomes, disease severity and parasite transmission (Lafferty, 2009; Shocket et al., 2018). This becomes particularly relevant in the context of climate change, which is causing significant fluctuations in global average temperatures.

In parallel, another crucial modulator of host-parasite interaction is coinfection. Since the late 1990s, several researchers have highlighted the role of coinfections in animals and plants (Petney & Andrews, 1998; Cox, 2001), suggesting that this is likely to be the rule rather than the exception in nature. Coinfections occur when two or more species of parasites – or different genotypes of the same species – coexist in a single host (Cox, 2001). The number of

studies using two or more parasites has increased significantly in recent years, however, knowledge about the effect of external variables such as temperature on the outcome of coinfection is still in its infancy.

The effect of temperature on coinfection may occur through its isolated effects on host and parasites as stated above. Additionally, temperature could potentially modify within-host interaction between parasites (Sardanyes et al., 2022), but empirical evidence is lacking. An example of temperature effects on coinfections was demonstrated in a plant-pathosystem. Alcaide et al. (2021) found that increasing temperature alters within-host competition between *Pepino mosaic potexvirus* in tomato plants, influencing virus genetic variability with possible consequences to evolutionary dynamics of viral populations. In another plant-pathogen system, mild temperatures were found to favor the development of the fungal parasite *Erysiphe quersicola* in the oak powdery mildew disease complex (Marçais et al., 2017). A wide variety of parasites can be part of coinfections and the diverse and complex within-host interactions they engender can be affected by temperature Thus, a better understanding of temperature effects on coinfection is crucial for advancing our knowledge in disease ecology.

In this chapter, we investigated the effect of temperature on coinfection outcomes in our model system composed by the host *Tenebrio molitor* and the fungi parasites *Beauveria bassiana* and *Metarhizium anisopliae*. These two fungi have similar mechanisms of infection and host exploitation strategies (see Hajek and St. Leger, 1994). This can attenuate the influence of parasite identity or biology on the outcome of coinfections and allows us to focus experimentally on temperature effects. Additionally, these fungi have different spore colors – white and green, respectively – and this makes their differentiation possible visually under a stereomicroscope, which is far simpler and cheaper than sophisticated and expensive molecular techniques, such as real-time PCR. This model system also allows us to check the impact of temperature on coinfection on the parasite side (see chapter 1). Coinfections are typically investigated in a *host-centric* perspective, with the consequences observed in terms of mortality or reproductive reduction (eg. Souza et al., 2019). However, considering the parasite side expands our understanding to the others players involved on coinfections.

Our previous results showed that *Beauveria* is a superior competitor compared to *Metarhizium* in coinfections when the fungi were inoculated simultaneously, and hosts were mantained at 25±1°C. In this chapter, our aim is to understand how temperature might affect this outcome. To do this, we conducted two experiments *in-vitro* with *Beauveria* and

Metarhizium – examining spore germination and radial *growth* – to assess their performance in isolation and in coexistence across a temperature gradient (18, 22, 26 and 30°C). Afterwards, we evaluated the *in-vivo* performance of hosts subjected to coinfection within the same temperature gradient, examining which parasite will dominate dead hosts. Through both *in vitro* and *in vivo* experiments, we aim to elucidate the interaction patterns between these entomopathogenic fungi and determine the influence of temperature on host-parasite and parasite-parasite interactions in our system.

2. MATERIAL AND METHODS

2.1 Insect colony

Larvae of *Tenebrio molitor* L. were reared in opaque plastic boxes (21×17×9 cm) with a ventilated lid at room temperature 25±1°C, 12:12 L:D photocycle, and 65 ± 10% relative humidity. Larvae were supplied *ad libitum* with a diet composed of a mix of oat bran, wheat fiber and wheat bran (1:1:1). Five slices of carrot were provided every 4 days. Boxes with larvae were checked twice a week and individuals which had pupated were manually transferred to new plastic boxes and covered with a sheet of A4 paper to allow emerged adults to hide. After adult emergence, they were transferred and maintained in plastic containers (41×26×7 cm) with the same diet plus carrot slices. Once a month, newly emerged larvae and eggs were collected.

2.2 Fungal suspensions

The fungal isolates *Beauveria bassiana* (B0265) and *Metarhizium anisopliae* (S2107) were used in all experiments (Costantin., 2024 chapter 1). For simplicity, we will refer to these as *Beauveria* and *Metarhizium* throughout the remainder of the text. Fungal suspensions were prepared by flooding fungal cultures cultivated on PDA (Potato Dextrose Agar) plates with 10ml of 0.01% Tween 80 and scraping the conidia with a Drigalski spatula. The suspensions were then stirred for 1 min. and filtered using sterile gauze. This procedure allowed the separation of the conidia from hyphal fragments. Conidial concentrations were estimated in a Neubauer Improved Chamber and adjusted to the desired concentration through serial dilutions. The suspensions were stored at 5°C and conidial viabilities were determined one day before the experiment. For this, an aliquot of 100µl of the suspension was individually plated on BDA culture medium and incubated at 25°C for 18 h. Suspensions were only used if they reached 95% germination. Stock concentrations were estimated using a Neubauer Improved Chamber and ten-fold serial dilutions were prepared and adjusted to the dose 1×10^6.

2.3 Germination experiment

Since spore germination represents the initial stage of the infection process for entomopathogenic fungi, we conducted an *in vitro* germination experiment to evaluate any

indication of inhibition in both if *Beauveria* and *Metarhizium* during coinfection. Single and mixed spore suspensions were prepared following 2.2 (above) at 1×10^8 conidia ml^{-1}. We inoculated 100µl of single and mixed suspensions in 15 × 60 mm Petri dishes containing only agar. We opted for agar as the medium to avoid the influence of nutrient media on fungal germination. The inoculation treatments were single *Beauveria*, single *Metarhizium*, co-inoculation of *Beauveria* and *Metarhizium*. The plates were then assigned to four different temperatures, 18, 22, 26 and 30°C, with 3 replicates in each treatment (n=3). The plates were incubated for 15 hours at each temperature and percentage germination was estimated checking the germination of 100 conidia under an optical microscope (at 400x magnification).

2.4 Single and Co-cultured paired bioassay

We assessed the performance of *Beauveria* and *Metarhizium in vitro* through single and paired bioassays conducted across different temperatures. Our idea was to determine whether there are differences in growth and interaction of both isolates when inoculated individually or together on culture medium. To to this, we individually inoculated 10µl at 1×10^6 conidia ml^{-1} of *Beauveria* and *Metarhizium* 5 mm from the border of Petri dishes (90 × 15 mm) containing PDA. In the paired trial, one fungus was inoculated on one side of the Petri dish, while the other was inoculated on opposite side, also at 5 mm from the plate edge. The experimental design was the same reported at 2.3. Each treatment had 10 Petri dishes as repetition (n=10). Fungal growth was evaluated every 4 days during 22 days. However, we conducted the analysis using the data from day 22, the last day of our experiment, when the fungi grew and occupied the entire plate. We photographed the plates using a digital camera (Nikon D 2000) and measured the colony radius growth using ImageJ 1.49v software.

2.4 Survival experiment and mycosis

The effect of temperature on the outcome of coinfection *in vivo* was investigated in a survival experiment. One microliter of mixed *Beauveria* and *Metarhizium* suspension at a concentration of $1x10^6$ spores ml^{-1} was topically inoculated on to the second thoracic segment of *T. molitor* larvae using a 2µl manual pipette. Each insect was observed until the liquid component of the suspension had been absorbed completely by the insect's cuticle (chapter 1). If the suspension came into contact with the Petri dish or the filter paper, the larva was replaced as we felt the total dose may have been reduced in these cases. After inoculation,

each larva larva was placed in individualized 15 × 60 mm Petri dishes, lined with filter paper and moistened with 200µl of sterile water. The plates were sealed with cling film and placed in incubators set at four different temperatures 18, 22, 26 and 30°C. Mortality was assessed daily for 12 days. After death, the insects were placed in a humid chamber (15 × 60 mm Petri dish, lined with filter paper and moistened with 250µl of sterile water) in an incubator (25 +/- 1°C) to stimulate fungal sporulation. Only larvae that presented signs of fungal sporulation were included in the analysis. We used n=20 *T. molitor* larvae (100-140mg) per treatment.

2.6 Statistical analysis

Germination and growth data were analyzed using a generalized linear model (GLM) assuming a quasi-binomial distribution for data that were overdispersed. Tukey *post hoc* tests was used to compare and estimate differences between treatments. Survival curves were fitted through Kaplan-Meier analysis to estimate host survival curves and Median Lethal Time (LT50). Cox proportional hazard (PH) model was used to determine the effect of temperature on survival time. Model simplification by backward stepwise deletion was carried out to amalgamate levels. Percentage of mycosis data were analyzed using a generalized linear model (GLM) assuming a quasi-binomial distribution. After only significant terms persisted in the model, further simplification was done by amalgamating levels. Models were compared using F-tests ($p < 0.05$).

3. RESULTS

3.1 Germination experiment

Spore germination was evaluated in single and co-inoculum at 18, 22, 26 and 30°C. Temperature had an effect on spore germination ($F_{11,24}$=11.788, p<0.0001). *Post hoc* tests showed that *Beauveria* had higher germination rates than *Metarhizium* in single inoculum at 22°C (p=0.0014), 26°C (p=0.0008) and 30°C (<.0001). In co-inoculum, *Beauveria* germination rate was higher than *Metarhizium* only at 22°C (p=0.0002). The inhibition of spore germination comparing single and co-inoculum only ocurred with *Beauveria* at 26°C (p=0.010)

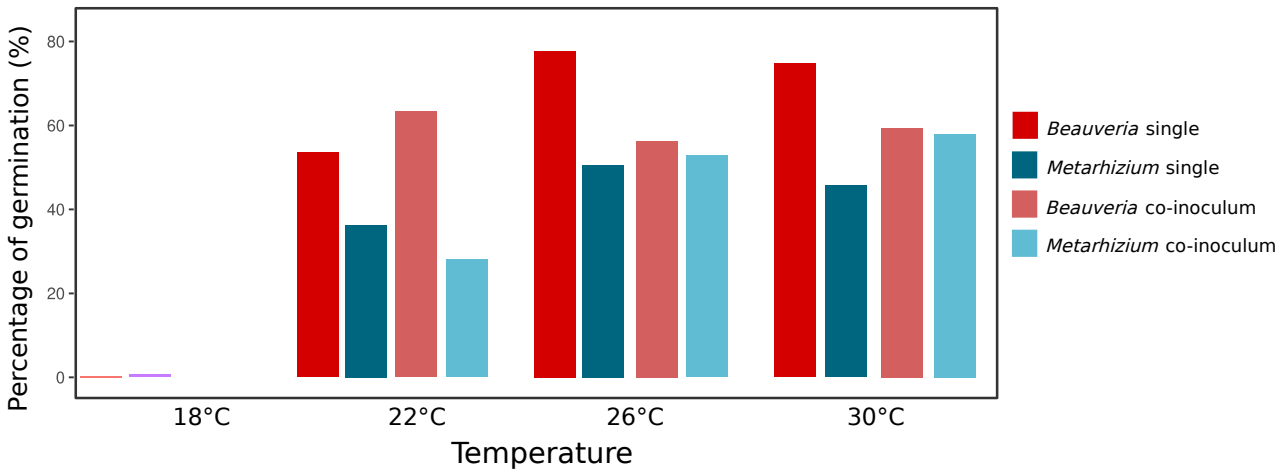

Figure 1. Germination of *Beauveria bassiana* and *Metarhizium anisopliae* in single and co-inoculum *in-vitro* at different temperatures. The fungi were inoculated individually or together in Petri dishes (15 × 60 mm) containing PDA, n=3. Plates were photographed after 15 hours and % of germination was estimated checking the germination of 100 conidia under an optical microscope. Tukey post hoc tests were used for pairwise comparisons.

3.2 Single and Co-culture bioassay

Colony growth was evaluated at 18, 22, 26 and 30°C in single and co-culture bioassays. Temperature had an effect on colony growth ($F_{15,133}$=29.995, p<0.0001) and *post hoc* tests showed that at 26°C *Beauveria* had higher growth than *Metarhizium* in single culture (p= 0.0050) and at 30°C *Metarhizium* had higher growth than *Beauveria* in single (p<0001) and co-culture (p=0.0243). However, at 18 and 22°C there was no difference between the colony radius of *Beauveria* and *Metarhizium* in single and co-culture (18°C – *Beauveria* vs. *Metarhizium* single culture p=0.9998; *Beauveria* vs. *Metarhizium* co-culture p=1.0000; 22°C - *Beauveria* vs. *Metarhizium* single culture p=1.0000; *Beauveria* vs. *Metarhizium* co-culture p=0.9998).

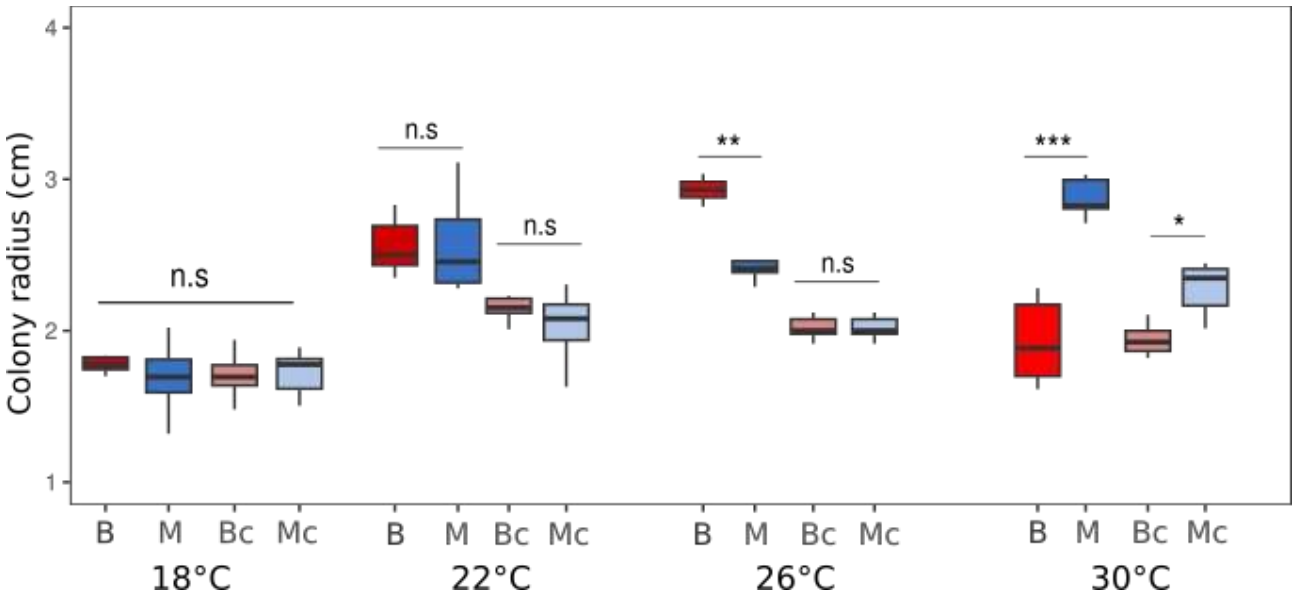

Figure 2. Growth of *Beauveria bassiana* and *Metarhizium anisopliae* in single and co-inoculum *in-vitro* at different temperatures. The fungi were inoculated individually or paired in Petri dishes (90 × 15 mm) containing PDA, n=10. In the paired trial, we inoculated one fungus in one side and the other fungus was inoculated on the side of the petri dish, also at 5 mm from the plate edge. Plates were photographed and colony radius was measured after 22 days. Tukey post hoc tests were used for pairwise comparisons (*P < 0.05, **P < 0.01, ***P < 0.001).

3.3 Survival experiment and mycosis

The percentage of survival of coinfected *T. molitor* larvae differed between the temperature gradient ($\chi^2_{[7]}$= 139.38, p<0.0001). Larvae on 18, 22 and 30°C had similar LT50 – 9.45 ± 0.36 days, 8.17 ± 0.54, 7.05 ± 0.60 days ± se, respectively ($\chi^2_{[5]}$= 7.239, p=0.0793). However, larvae on 26°C died faster than the other temperature treatments LT50- 5.94 ± 0.20 days ($\chi^2_{[4]}$= 112.45, p=<0.0001).

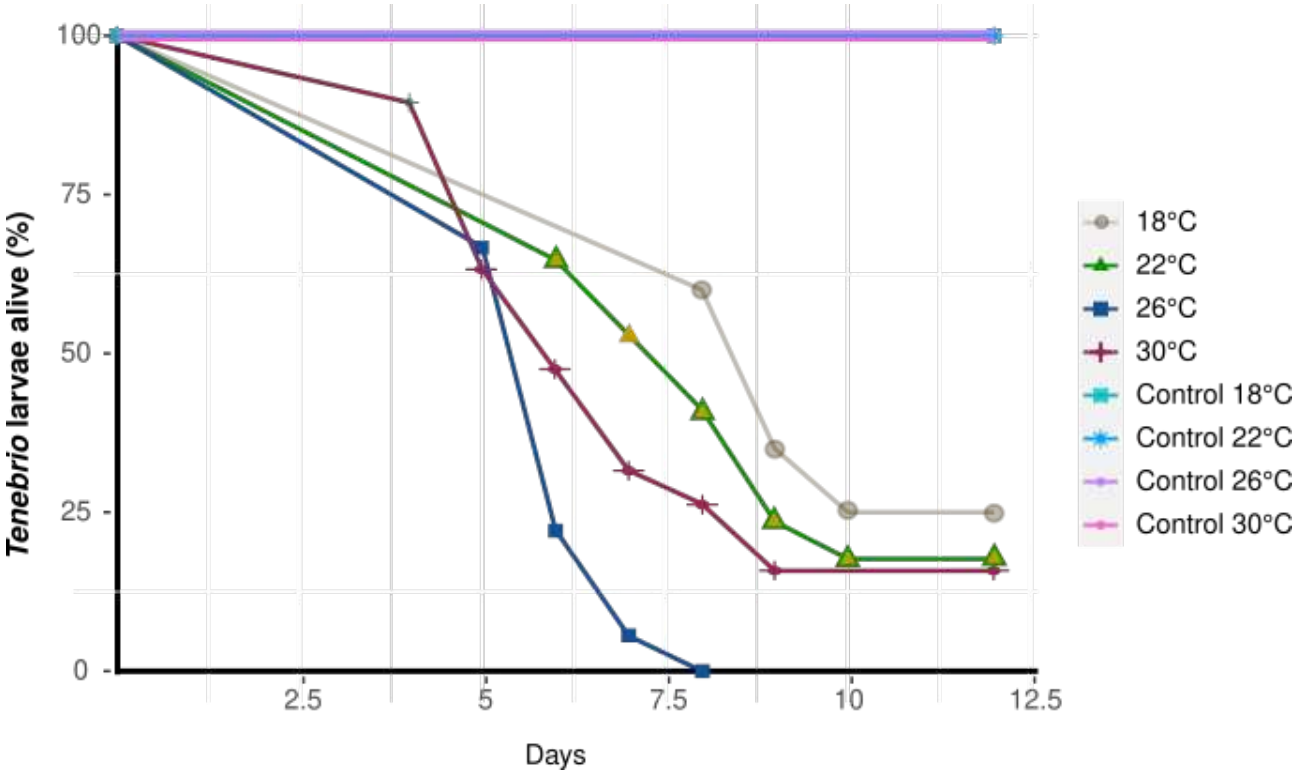

Figure 3. Cumulative survival *Tenebrio molitor* larvae exposed to coinfections between *Beauveria bassiana* and *Metarhizium anisopliae* in different temperatures. The insects (n=20) were topically inoculated with 1µl of mixed fungal suspensions (1×10^6 spores ml^{-1}) and placed in incubators at four temperatures 18, 22, 26 and 30°C. Diet was replaced daily and larval mortality was assessed daily during 12 days.

The percentage of hosts colonized only by *Beauveria* was similar at 18, 22 and 26°C - 100, 75 and 66.6%, respectively ($F_{1,61}$=1.268, p=0.264). However, *Beauveria* dominance was diminished at 30°C, where 50% of the hosts were dominated by this fungus ($F_{1,61}$=4.732, p=0.0335).

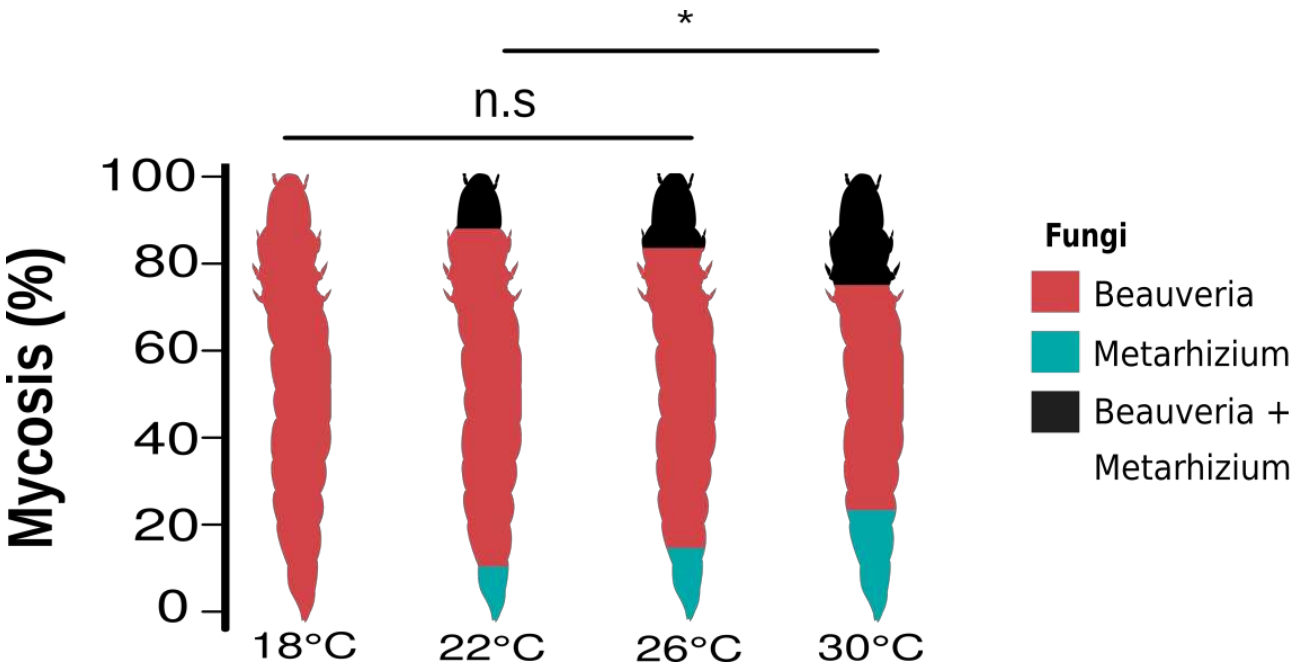

Figure 4. Percentage fungal sporulation in *Tenebrio molitor* larvae with single and mixed infections of *Beauveria bassiana* and *Metarhizium anisopliae*. After death, insects were places in a Petri dish lined with moistened filter paper and the percentage of dead larvae in which a single fungal species or both sporulated was observed after 25 days in humid chamber. (*P < 0.05, **P < 0.01, ***P < 0.001).

4. DISCUSSION

Temperature is a well known factor driving host-parasite interactions, with implications for disease severity, transmission and epidemics (Shocket et al., 2018, Rorh and Cohen, 2020). Nevertheless, our understanding of effects of temperature on coinfections remains limited. Using two parasitic fungi – *Beauveria* and *Metarhizium* – as a model system, we investigate how coinfection outcomes may vary with temperature and the resulting consequences for hosts and parasites. Our study involved both *in-vitro* and *in-vivo* tests to assess the performance of both parasites in isolation and in a co-inoculum.

A variety of biotic and abiotic factors can inhibit spore germination in fungi, including the presence of a competitor and temperature. Therefore, we performed the germination experiment because entomopathogenic fungi need to attach and penetrate the insect cuticle to infect their hosts, and competition may begin at this stage (Li et al., 2021). Our results showed that *Beauveria* exhibited higher germination rates than *Metarhizium* when inoculated alone at 22, 26 and 30°C. However, in co-inoculum, the germination rate of *Beauveria* was only superior at 22°C. We also did not observe a strong indication of spore inhibition in co-inoculum. The percentage of germination in this group was only different from single inoculum for *Beauveria* at 26°C. These results indicates that, overall, both fungi exhibited similar germination rates in co-inoculum, suggesting that germination alone may not explain why *Beauveria* is a superior competitor in coinfections across the tested temperature range. However, it is important to consider other processes involved in the initial infection stage, such as appressorium formation or secondary metabolites that have roles in infection (Almudena Ortiz-Urquiza and Keyhani, 2013), which also may be impacted by temperature and could represent an advantage to *Beauveria.*

The *in vitro* growth of parasitic fungi could be an indication of parasite performance. If a fungus grows faster it can dominate the infection process and overpower the competitors (Jensen et al., 2016). At 26°C, *Beauveria* grew faster than *Metarhizium* when inoculated alone, while the opposite was observed at 30°C. This suggests that the thermal *optimum* for *in vitro* growth is different for each fungus, potentially leading to different performance *in vivo*. We indeed found that *Beauveria* was the dominant fungus at 18, 22 and 26°C, with 100%, 75% and 66.66% of dead insects being colonized only by *Beauveria*, respectively. However, at 30°C this dominance was significantly reduced – *Beauveria* was the only sporulated fungus in 50% of insects. While the *in vitro* result of *Metarhizium* being the faster-growing fungus at

30°C helps to explain the reduction in *Beauveria's* dominance, it appears to be insufficient to make *Metarhizium* the most dominant fungus in coinfection, contrary to our prediction. For instance, competition through the production of toxic metabolites, known as a fungal defensive strategy (Kempken and Rohlfs, 2010; Künzler, 2018), could be involved. Additionally, the specificity of interaction of each fungal species with the host immune system may play a significant role in this outcome. Further experiments investigating host immune response and metabolite production by these fungi could provide more information about the interaction between *Beauveria* and *Metarhizium* and how this influence the outcome of coinfection in this system.

On the host side, larval mortality was higher at 26°C compared to the other temperatures. This result can be explained by the thermal performance curve of the host and the parasites involved in the infection (Mólnar et al., 2017; Padfield et al., 2020). It is commonly reported that entomopathogenic fungi exhibit their highest growth rates within the range 25-30°C (Ekesi et al., 1999; Davidson et al., 2003; Polar et al., 2005). Consistent with this, our study found that *Beauveria* growth peaked at 26°C, while *Metarhizium* growth peaked at 30°C. However, it is noteworthy that insects in general tend to have an optimum temperature range for growth and development that exceeds 30°C. For example, Bjørge et al (2018) demonstrated that the growth of two species of beetle, *Alphitobius diaperinus* and *Tenebrio molitor,* reached their maxima at 31 °C. The disparity between host and parasite thermal performance curves could explain the higher mortality rate observed at 26°C; this temperature falls within the higher growth performance range for the fungi, but outside the optimum range for the host. Additionally, the within-host interaction between *Beauveria* and *Metarhizium* was altered at 30°C. The increased growth rate of *Metarhizium* and the reduced dominance of *Beauveria* could be indicative of intensified competition between them. In this situation, a shift of fungal resources from virulence factors to competitor inhibition factors might benefit the host.

Our model system has proven to be useful to evaluate the impact of another abiotic factor and how it could impact coinfections. Together, our findings demonstrate that temperature can change the outcome of coinfections and hosts and parasites suffer its effects. Host mortality was highest at 26°C which is the most suitable temperature for the development of entomopathogenic fungi and does not correspond to the *optimum* temperature range for hosts such as *T. molitor*. However, at 30°C host mortality began to decrease, which

seems to be closer to the host peak of growth and development. Moreover, the within-host interaction also changed at the warmer temperature. The dominance of *Beauveria* started to decrease at 30°C, consistent with the *in vitro* result showing that *Metarhizium* performs better at this temperature.

An ongoing change in global temperature will affect species interactions with consequences for parasitism (Paull et al., 2012; Barber et al., 2016). Understanding the magnitude of these changes poses a challenge for research in this field, and our results suggests that both host-parasite and parasite-parasite interactions can be equally affected by temperature, thereby explaining changes in disease outcomes. In an applied context, *Metarhizium* and *Beauveria* are the most used fungi in biological control of insects in the world, and the mixture of these agents has been considered to overcome some inconsistencies in control (Inglis et al., 1997). Our results suggest that insect control using fungi could be compromised under high temperature conditions. The decrease in host mortality at elevated temperatures indicate potential challenges for effective insect control strategies in warmer environments. In an ecological context, such changes ultimately have the potential to affect parasite and host populations (Altizer et al., 2013), disease prevalence and epidemics (Mora et al., 2022).

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FINAL CONSIDERATIONS

Despite the increase in the number of studies on coinfections, they remain poorly understood. There are still still many gaps in research that covers coinfections. Firstly, it is their impact on the host's fitness that is predominantly studied, leaving the parasites' fitness, a fundamental component, to one side. Secondly, the results of coinfections between different species are still quite unpredictable; this is partly because work in this area uses parasites with very different taxonomic identities (eg. fungi and bacteria); which makes it difficult to draw generalizations and test the related theory. Finally, due to the unpredictability of coinfection outcomes, little is known about the characteristics of infections, environmental factors, and the immune responses of the hosts that may impact them. These three characteristics make the studies in this field unresolved and mean that there have been few theoretical and practical advances. This thesis aimed to address some of these problems. Through our study system, we were able to test several variables that could impact coinfections outcomes, such as characteristics of infection, host nutrition and temperature. These impacts could be measured for both hosts and parasites giving a more complete view of environmental effects on coinfections. We began here to build a body of theory in one type of system that can be considered to other biological systems. Given the peculiarities of each system, researchers will be able to select a set of variables to be investigated in their work. Finally, the two selected fungi are the most used in biological control of insects in Brazil and the world, including in mixtures, and understanding how they interact will help to develop more rational pest control strategies, aiming for better efficiency of biological products in agriculture.