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Within-host competition during fungal coinfection in an insect host

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

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ABSTRACT

LIMA, Nathan Lemes da Silva, M.Sc., Universidade Federal de Viçosa, February, 2026. **Within-host competition during fungal coinfection in an insect host**. Adviser: Simon Luke Elliot. Co-advisers: Eduardo Carlos Costantin and Thairine Mendes Pereira.

Coinfections are common in natural populations and can strongly alter parasite fitness due to within-host competition and host exploitation. Yet, general mechanisms remain difficult to infer because many coinfection studies involve parasites that differ widely in life history and within-host resource use. Here, we used a tractable insect–fungus system in which two ecologically comparable entomopathogenic fungi (Metarhizium spp. and Beauveria bassiana; Hypocreales) coinfect the host Tenebrio molitor (Coleoptera: Tenebrionidae), allowing competition to be assessed under a shared infection niche. In Chapter 2, we quantified isolate-level variation in virulence, host colonization, and spore production using ten Metarhizium isolates in coinfection with a reference B. bassiana isolate. Metarhizium virulence varied widely among isolates and was strongly conserved between single and coinfections. More virulent isolates were more likely to colonize hosts during coinfection, explaining a substantial proportion of variation in competitive success. In contrast, virulence showed weak or no association with spore production once colonization was achieved. A multivariate ordination of coinfection outcomes further revealed that saprophytic sporulation on rice (taken as a proxy for post-mortem growth) structured the multivariate pattern of competitive outcomes. In Chapter 3, we provide preliminary data on a comparative histopathological assessment of single and coinfections. Fungal structures were most consistently associated with the fat body. However, a high-virulence Metarhizium isolate showed more extensive tissue occupation, including fungal structures in muscle tissue. Together, the empirical chapters show that coinfection outcomes are governed by stage-specific components of fungal fitness, with some initial indications of relevant mechanistic aspects.

Keywords: host-parasite interactions; entomopathogenic fungi; within-host competition; parasite fitness; Metarhizium; Beauveria

RESUMO

LIMA, Nathan Lemes da Silva, M.Sc., Universidade Federal de Viçosa, fevereiro de 2026. **Competição intra-hospedeiro durante coinfecção fúngica em insetos**. Orientador: Simon Luke Elliot. Coorientadores: Eduardo Carlos Costantin e Thairine Mendes Pereira.

Coinfecções são comuns em populações naturais e podem alterar o fitness dos parasitas devido à exploração e competição dentro do hospedeiro. No entanto, mecanismos gerais ainda são difíceis de inferir, pois muitos estudos de coinfecções envolvem parasitas com diferentes histórias de vida e modo de exploração do hospedeiro. Aqui, utilizamos um sistema inseto-fungo no qual dois fungos entomopatogênicos ecologicamente comparáveis (Metarhizium spp. e Beauveria bassiana; Hypocreales) coinfectam o hospedeiro Tenebrio molitor (Coleoptera: Tenebrionidae), permitindo que a competição seja avaliada sob um nicho de infecção comum. No Capítulo 2, quantificamos a variação da virulência ao nível de isolado, a colonização do hospedeiro e a produção de esporos utilizando dez isolados de Metarhizium em coinfecção com um isolado de referência de B. bassiana. A virulência de Metarhizium variou amplamente entre os isolados e foi conservada entre infecções simples e coinfecções. Isolados mais virulentos apresentaram maior probabilidade de colonizar os hospedeiros durante a coinfecção, explicando uma proporção substancial da variação no sucesso competitivo. Em contraste, a virulência mostrou associação fraca ou inexistente com a produção de esporos após a colonização ser estabelecida. Uma ordenação multivariada dos desfechos de coinfecção revelou ainda que a esporulação saprofítica em arroz (utilizada como proxy para o crescimento pós-morte) estruturou o padrão multivariado dos resultados competitivos. No Capítulo 3, apresentamos dados preliminares de uma avaliação comparativa da histopatologia das infecções simples e coinfecções. Estruturas fúngicas estiveram mais consistentemente associadas ao corpo gorduroso. No entanto, um isolado de Metarhizium de alta virulência apresentou ocupação tecidual mais extensa, incluindo o tecido muscular do hospedeiro. Em conjunto, os capítulos empíricos mostram que os desfechos de coinfecção são governados por componentes específicos durante os estágios da doença, com algumas indicações iniciais sobre mecanismos relevantes.

Palavras-chave: interação parasita-hospedeiro; fungos entomopatogênicos; competição dentro do hospedeiro; fitness; Metarhizium; Beauveria

SUMMARY

GENERAL INTRODUCTION

1.1 Coinfections

Host-parasite relationships are highly intimate interspecific interactions (De Leo et al. 2002) as parasites are organisms that live at the expense of their hosts. This symbiotic association is detrimental to hosts, as parasite fitness completely depends on using host resources to complete its life cycle. The damage inflicted is observed as disease, which is defined as a departure from the state of health or normality (Casadevall and Pirofski 2000; Hajek and Shapiro 2018). This host-parasite association is central to community and population ecology (Bonsall 2004), and can be influenced by environmental factors, symbiotic associations, and competition among coinfecting parasites (Atique et al. 2024).

Traditionally, host-parasite interactions have been studied through systems of a single parasite infecting a single host. These one-host, one-parasite research models have been instrumental in understanding the life cycle of parasites, their detrimental effects on host fitness, and their transmissibility (Costantin 2025). However, such models oversimplify the host-parasite interactions, as, in nature, hosts can be simultaneously infected by multiple parasite species or different genotypes of the same species. Such concomitant infections are widespread (Read and Taylor 2001), as a single host can represent an ecosystem in which different parasites interact. For example, approximately 30% of infections in humans may involve coinfections, and this rate could reach up to 80% in some human communities (Vaumourin et al. 2015). These coinfections can impact the host's fitness and the dynamics of each infecting parasite (Rynkiewicz et al. 2015).

From the host perspective, coinfections have an extensive range of effects and often result in more pronounced consequences on the host's fitness than single infections, primarily due to interactions between coinfecting parasites (Alizon et al. 2013; Graham et al. 2007). These interactions can have negative consequences on the host such as accelerated death (Vaumourin et al., 2015). For example, the coinfection of gastrointestinal nematodes and *Mycobacterium bovis* accelerates mortality in African buffaloes (Jolles et al. 2008). However, negative effects of coinfection towards the host can be reduced through antagonistic interactions between infecting agents, as occurs in frogs coinfected with the helminth *Echinoparyphium* and

Ranavirus: these coinfected individuals exhibit a lower viral load than individuals infected only by the virus, suggesting a possible cross-reactive immunity (Wuerthner et al. 2017). This reduction in viral replication represents an antagonistic relationship between parasites, although this interaction does not necessarily represent improvement for the host fitness.

For parasites, coinfections can also affect performance, with interactions ranging from beneficial to antagonistic. Cooperative interactions may occur when parasites indirectly benefit one another through the production of public goods, such as metabolites driving easier host resource exploitation. Meanwhile, neutral interactions occur when the presence of one parasite does not impact any aspect of the other one (e.g. growth, virulence or fitness). Antagonistic interactions arise when one parasite suppresses the establishment of its competitor, whether through resource competition, immune-mediated interference or inhibitory metabolites (Tanada 1976). These interactions may determine the outcomes of coinfections, but their relationship and potential trade-offs remain unclear (Sheng and St. Leger 2024).

1.2 Parasite strategies within coinfection

During coinfections, parasites are under pressure from host defenses and interactions with the competing parasite. In these conditions, parasite fitness can be shaped by how efficiently each parasite exploits the host's resources while dealing with competition from other infecting agents. Consequently, parasites can adopt different exploitation strategies that influence their success, for example, better growth rates, virulence, and transmission within a coinfected host.

Obligate killing parasites are parasites whose life cycle requires the death of the host to complete transmission – they allocate host resources to the production of infective stages, which are released after or as a direct consequence of host death (Ebert and Weisser 1997). As transmission depends on killing the host, obligate killing parasites face a fundamental trade-off between within-host growth and timing of host death. In this situation, killing the host too early can limit parasite replication and reduce the number of transmission stages produced, whereas delaying host death allows greater exploitation of host resources. This trade-off strongly shapes parasite strategies of growth and virulence in single infections, consistent with the concept of 'first grow then kill' strategy in which parasites delay host death until sufficient replication has occurred to maximize propagule yield. However, in coinfections, a single host is a limited

resource that multiple parasites can compete for (Rigaud et al. 2010), and in this context, a parasite's faster exploitation strategy should overcome more prudent strategies. Thereby, although they favor parasites that cause more significant damage and higher mortality (van Baalen and Sabelis 1995), these competitive relationships carry tradeoffs. For example, the one replicating most vigorously gains a fitness advantage and dominates the host, but this rapid parasite replication may lead to the host's premature death, reducing overall transmission rates (Bremermann and Pickering 1983; Levin 1983; Nowak and May 1994).

The competitive ability of each pathogen is linked to the parasite's capacity to optimally utilize host resources for growth and reproduction while coexisting with other parasites (Kershaw et al. 1999). However, its success in reproducing, for instance, also depends on the strategy imposed by other potential competitors. For example, empirical studies concerning multiple infecting fungal strains in insects suggest a pattern of competitive exclusion, with no evidence of synergistic effect between the strains (Li et al. 2021). A parasite's ability to outcompete another or to complete its life cycle in the presence of a competitor is not necessarily correlated with virulence, which is defined here as parasite-induced mortality (Bell et al. 2006; Staves and Knell 2010). However, a resource utilization strategy is employed by many intrinsic mechanisms of a given microorganism, and it may confer competitive advantages. For example, entomopathogenic fungi in the genus *Metarhizium* have evolved numerous mechanisms that enable them to attack, parasitize, and gain nutrients from insects (St. Leger and Wang 2020).

Competition between parasites can occur through three mechanisms (Mideo 2009), which influence parasite strategies for survival, growth, or reproduction. Exploitation competition arises when parasites compete directly for limited host resources, such as nutrients or host tissues, in which increased resource use by one parasite reduces availability for the other. An example is the leaf-roller moth *Adoxophyes honmai*, the tea tortrix, coinfected by *Alphabaculovirus adhonmai* and *Betaentomopoxvirus ahonmai*. In this situation, the virus that succeeds in replicating faster has an advantage in acquiring host resources early and significantly reduces the viral load of its competitor (Ishii et al. 2002). The second, immune-mediated apparent competition, occurs when a parasite alters the host's immune responses and indirectly suppresses the performance of a coinfecting parasite. In this case, the parasites do not compete for resources directly, but immune responses elicited by one parasite negatively impact the other one. For example, in human malarial infections, the avirulent species *Plasmodium vivax* elicits the host immune system and offers protection against the virulent species

*Plasmodium falciparum (*Maitland et al. 1997). Lastly, interference competition involves direct inhibition of competitors' growth, reproduction, or transmission, through the production of inhibitory compounds or physical exclusion. This kind of competition can occur through secondary metabolites produced by entomopathogenic fungi that act as antimicrobial agents (Zhang et al. 2020).

Coinfection study systems introduce an additional layer of complexity to the ecological interactions between hosts and parasites, as these concomitant infections influence disease aspects by positively or negatively altering the host's and the parasites' fitness. These interactions are complex because the burden of one or both parasites may increase, one or both may be suppressed, or one may increase while the other is suppressed (Cox 2001). This diversity of outcomes is linked with parasite strategies and competitive mechanisms operating within hosts and implies that coinfections significantly alter the population dynamics of the interacting parasites and their effects on the host's fitness (Thomas et al. 2003). As a result, understanding coinfections requires experimental systems that allow direct study of how specific parasite traits translate into competitive success.

1.3 Tenebrio-Beauveria-Metarhizium model system

Most studies of coinfections involve parasites (often of different kingdoms) that differ considerably in life history, infection route, or host resource use, making it difficult to understand general mechanisms of the coinfecting agents (e.g. Graham 2008; Telfer et al. 2010). To address this limitation, in the *Tenebrio-Beauveria-Metarhizium* model system established by Costantin et al. (2025), the competing parasites (*Beauveria bassiana* vs. *Metarhizium robertsii*) are of the same fungal order (Ascomycetes: Hypocreales) and are ecologically comparable, particularly because they share similar ecological traits and occupy overlapping within-host niches, allowing the investigation of ecological aspects beyond parasite identity.

On the host side, vertebrate and invertebrate models have been used for the investigation of microbial infections (Brunke et al. 2015). Insects are frequently used as invertebrate models (Arvanitis et al. 2013) to study disease. For example, the fruit fly, *Drosophila melanogaster*, has been widely used to investigate replication of human viruses or bacterial virulence (Hughes et al. 2021; Pimentel et al. 2021; Buchon et al, 2014). Similarly, the beetle *Tenebrio molitor* (Coleoptera: Tenebrionidae) provides several advantages as an experimental host model as it

can be easily reared and maintained under laboratory conditions, mainly due to the low costs associated with its mass rearing, such as diet it is also sensitive to infection by a wide range of micro-organisms - when infected by fungi, as is the case here, it becomes an excellent example, as the development of the disease can be well observed. Beyond its use as a laboratory model, from a practical perspective, the yellow mealworm is considered a pest of stored grain products and is also one of the most promising insects for human nutrition (Noyens et al. 2024).

On the parasites' side, entomopathogenic fungi are useful models to study coinfections as they naturally co-occur, infecting the same host and exhibiting measurable competitive interactions within the host environment. In particular, *Beauveria* and *Metarhizium* are cosmopolitan generalist pathogens that share similar ecological traits and infection strategies, which makes them well-suited for exploring parasite-parasite interactions. The lifecycle of these fungi, described briefly below, is broadly studied and well described, which allows us to associate distinct infection phases with strategies employed during the within-host interactions.

The infection of insects by entomopathogenic fungi begins with the initial attachment of conidia to the insect cuticle. Conidia become metabolically active and germinate by producing a germ tube, and the penetration involves an enzymatic activity complex of chitinases, lipases, and proteases. Structural adaptations can also be involved to optimize the process, such as the formation of appressoria and the mechanical pressure by hyphae with the accumulation of cellular turgor pressure for cuticle penetration (Butt et al. 2016). Some species like *Beauveria bassiana* do not produce appressoria for cuticle penetration; this fungus degrades, penetrates, and assimilates the insect cuticle by combining many cuticle-degrading enzymes and mechanical pressure (Ortiz-Urquiza and Keyhani 2013; Shang et al. 2024). Once the cuticle has been breached, the fungi propagate in the host hemocoel, budding either in a yeast-like phase, the blastospores, or as hyphal bodies (St. Leger and Wang 2020) that can freely circulate through the insect. They reproduce by assimilating nutrients and evading antimicrobial proteins and hemocytes (Lu and St. Leger 2016). The fungi kill the insect through tissue disruption from hyphal invasion, nutrient depletion, and a diversity of mycotoxins (Gul et al. 2014). Upon host death, these fungi start to grow saprophytically with hyphae reemerging from the inside of the insect's body to cover the cadaver and produce massive numbers of conidia to infect new hosts.

When *Tenebrio* is infected by both *Beauveria* and *Metarhizium*, these fungal coinfections can be easily visualized through host post-mortem colonization, whether predominantly by one fungus or both sporulating. A major practical advantage is that the two

fungi differ in spore aspects and morphology - *Metarhizium* produces green rod-shaped spores and *Beauveria* white, rounded spores, making the host's colonization easily assessed. This easy spore differentiation allows us to quantify parasite fitness using two complementary metrics: colonization success – measured as the proportion of hosts colonized only by either of the fungi or by both fungi simultaneously (Fig. 1) – and the reproductive output – measured as spore yield per cadaver – in different coinfection outcomes (Costantin et al. 2025).

Because the coinfection outcomes can be quantified, this provides a simple and tractable measure of within-host competition. It allows us to evaluate how competitive interactions can be associated with successful host colonization and reproductive output.

In the first study of the *Beauveria-Metarhizium-Tenebrio,* Costantin et al. (2025) demonstrate that the first parasite to arrive dominates most infections, and that inoculation in different locations increases the proportion of hosts colonized by both fungi, although there is a reduction of parasite fitness (spore production) in these circumstances. In subsequent studies, Costantin (2024) shows that host nutrition (protein to carbohydrate ratio) had only a limited impact on coinfections and parasite fitness, while temperature strongly affects the outcomes of coinfections.

While many perspectives of coinfection research models are host-oriented, this model enables a complementary focus on parasite ecology, an important aspect that may shape the consequences of coinfection. The competitive success, host colonization, and parasite fitness must be inferred from measurable proxies such as fungal growth, tissue invasion, and reproduction, through complementary techniques representing biological processes.

Figure 1. Post-mortem sporulation outcomes of *Tenebrio molitor* larvae infected by *Metarhizium* (A) *Beauveria bassiana* (B). Coinfected cadavers showing simultaneous sporulation of both fungal species (C-D), arrowheads indicate species-specific sporulation on the cadaver surface, yellow arrowheads: *B. bassiana* and blue arrowheads: *Metarhizium*. Overall sporulation appears reduced when both fungi sporulate on the same host (C-D) relative to cadavers dominated by a single fungus (A–B). Photos: the author.

1.4 Methods to investigate fungal infections

Although coinfection outcomes can be quantified through post-mortem colonization patterns (Costantin et al. 2025 and Chapter 2 of this Dissertation), these patterns provide limited insight into the underlying mechanisms. For this, several methodological approaches can be used, ranging from simple morphological observations to advanced molecular approaches (Dunlap et al. 2017). Integrated approaches that quantify fungal development, host exploitation,

and within-host dynamics across infection stages can help reveal the mechanisms driving competitive dominance during coinfection.

Traditional morphological methods for identifying fungi focus on the macroscopic and microscopic examination of fungal structures (Piontelli 2015) such as conidia, hyphal bodies, mycelia, and conidiophores that can be observed in or on infected insects. For example, the shape and size of the conidia are the traits most used to identify *Beauveria* and *Metarhizium* species (Samson et al. 1988).

The main fungal strategy to invade a host is through chemical substances, which can be assessed by chromatography. This biochemical approach is related to enzymatic activity, such as the production of chitinase, lipases, and proteases (Sánchez-Pérez et al. 2014), as well as other secondary products, including metabolites. Furthermore, the chromatography method helps to understand fungal physiology, fungi-insect interactions, and within-host interactions towards disease progression. For example, the assessment of phospholipases corresponds to the capacity of the fungi to degrade phospholipids in the insect's cuticle (Moharram et al. 2021).

Once inside the host, entomopathogenic fungi start to spread and develop at the host's expense. Molecular methods have significantly enhanced the evaluation of fungal detection inside the host. The polymerase chain reaction (PCR) is a powerful methodology for detecting and identifying fungal species infecting a host (Schoch et al. 2012), and additionally, quantitative PCR (qPCR) can quantify fungal DNA in a sample (Arquiza-Apollo and Hunter 2014). This methodology offers a description of the infection intensity by considering the fungal population inside a host. The molecular approach alone does not replace the importance of using classical methods such as histological assessments of fungal development and tissue invasion. Both techniques complement each other to achieve an accurate and descriptive identification of the fungi in a host (Hyde et al. 2010).

The interaction between the parasite and host tissues represents the detrimental effects of host exploitation. To investigate this, histological study of the infection enables the visualization of disease progression in host tissues and fungal structures. This method is a critical primary data source for assessing tissue alterations, cellular abnormalities, and parasite presence (Lee et al. 2021). With a fixative, it is possible to preserve tissue integrity and fungal morphology at a given moment of the infection, and it consists of the sample sectioning using a microtome and a staining protocol of the thin slices to promote contrasts between host tissues

and fungal structures. Histology reveals details related to the fungal presence and its infective processes, such as proliferation and interactions within the host.

The integration of these many methodologies allows researchers to achieve a comprehensive understanding of entomopathogenic fungi in arthropod hosts. While traditional approaches remain essential for describing infection aspects, molecular and advanced methods can improve resolution regarding fungal presence and infection intensity. Here, we begin investigating the mechanisms underlying infection and competition in our system by focusing on histopathology, using tissue-based assessments to describe fungal establishment, within-host distribution, and host tissue alterations under single and coinfections.

1.5 This Dissertation

In this study, we aim to investigate how intrinsic parasite traits and within-host competitive mechanisms shape coinfection outcomes between entomopathogenic fungi. Using the *Beauveria–Metarhizium–Tenebrio* model system, the central objective is to determine whether fungal traits related to growth, virulence, and resource exploitation translates into differences in competitive ability and host colonization success during coinfection. By focusing on obligate-killing parasites that share infection routes and within-host niches, we link parasite ecology with disease outcomes in a controlled experimental framework.

To address these objectives, in Chapter 2, we adopt an ecological perspective, quantifying key components of parasite fitness under single and coinfections. We investigate how differences in virulence – time to kill the host – could determine competitive ability by providing earlier access to host resources and the cadaver as a substrate. Using ten isolates of *Metarhizium* versus a reference isolate of *Beauveria bassiana*, we assess isolate-specific variation in germination speed, virulence, saprophytic growth, and spore production, and test if competitive outcomes during coinfection can be predicted from performance in single infections. We found that the speed of conidia germination and virulence varies widely among the *Metarhizium* isolates (as expected), but there is no relation between these two traits. Virulence is strongly conserved between single and coinfection conditions, and more virulent isolates are more likely to colonize hosts during coinfection, in line with theory (see Section 1.3 above). In contrast with this, virulence explains little of the variation in spore production once colonization is achieved. Finally, multivariate analyses indicate that spore production on

rice (a proxy for saprophytic growth) structures coinfection outcomes, highlighting the importance of post-mortem performance for competitive dominance.

Chapter 3 is an initial characterization of how competing fungi establish and proliferate. Through histopathological analyses, we describe spatial patterns of fungal growth and tissue invasion under single and coinfections. We show that fungal structures are most consistently associated with the fat body, whereas muscle tissue is comparatively preserved in most infections. The infections involving a high-virulence *Metarhizium* isolate exhibit more extensive tissue occupation and clear evidence of fungal structures in muscle tissue compared to the low-virulence isolate, linking isolate-level virulence to contrasting within-host exploitation patterns. This chapter is a qualitative assessment that establishes the baseline for future quantitative analyses. We intend to quantify fungal tissue occupation as the area covered by fungal structures in the different treatments, characterize hyphal morphology and evaluate the host immune related responses, as hemocyte abundance and distribution.

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VIRULENCE DETERMINES COMPETITIVE SUCCESS DURING FUNGAL COINFECTION IN AN INSECT HOST

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ABSTRACT

When parasites coinfect a single host, they can occupy the same within-host niche and compete. In this competitive environment, infection success may depend on isolate-specific traits expressed at different stages of the infection cycle. However, the relative contribution of traits acting before and after host death to competitive outcomes remains poorly understood. Parasite isolates can differ strongly in virulence, growth, and exploitation strategies, yet it remains unclear whether such isolate-level traits can reliably explain which parasite dominates within-host competition. Here, we ask whether variation in competitive success during coinfection can be predicted from isolate-specific performance traits. Entomopathogenic fungi provide a useful model to address this question, as host death is followed by an extended phase of post-mortem growth and sporulation. Here, we investigated how variation in virulence and performance traits shapes competition during coinfection between *Metarhizium* and *Beauveria* in the insect host *Tenebrio molitor*. We quantified virulence, post-mortem host colonization, and spore production for ten *Metarhizium* isolates coinfecting hosts with a reference *Beauveria* isolate. Virulence varied widely among *Metarhizium* isolates and was strongly conserved between single and coinfection treatments. More virulent isolates were more likely to colonize hosts during coinfection, explaining nearly half of the variation in colonization success. In contrast, virulence showed a weak or no association with spore production once colonization was achieved. A multivariate ordination of coinfection outcomes revealed that saprophytic spore production on rice, a proxy for post-mortem growth, was the only trait significantly structuring competitive outcomes. Together, these results demonstrate that virulence determines competitive ability and that saprophytic performance shapes reproductive dominance after host death. Overall, this work supports a trait-based view of coinfections, showing that isolate-level differences can generate contrasting competitive strategies and predict coinfection outcomes.

Keywords: host-parasite interactions, fungal competition, *Metarhizium*, *Beauveria, Tenebrio molitor*

1 INTRODUCTION

The establishment of populations in new habitats and their further expansion requires individuals to cope with the ecological contexts of a habitat, including abiotic conditions and the presence of competitors (Alzate et al. 2020). In competitive environments, success can be shaped by intrinsic traits related to exploitation efficiency, growth, interference strategies, and pressure caused by the competitor (Kershaw et al. 1999; Staves and Knell, 2010). This ecological framework applies to host-parasite interactions, in which parasites face not only pressures of host defenses, but also the challenges of competing with other parasites in coinfections. In single infections, highly virulent exploitation strategies can result in the host's death before the parasite has made full use of available resources, limiting parasite replication and transmission, so selection may favour restraint (Bremermann and Pickering 1983; Levin 1983; Nowak and May 1994; van Baalen and Sabelis 1995; Pels and Sabelis 1999). In coinfections (infection by two or more parasites in a single host), parasites may interact and compete through antagonistic mechanisms that can alter the outcomes of the infection (Sheng and St. Leger 2024; Budischak et al. 2018). In this setting, higher virulence may be selected if it confers a competitive advantage, even though it may lead to lower overall fitness of the parasites (van Baalen and Sabelis 1995; Pels and Sabelis 1999). Thus, the relative success of each parasite can be tightly linked to its intrinsic characteristics (Mideo 2009; Knowles 2011; Wale et al. 2017; Ramiro et al. 2016). Parasite genotypes differ in virulence, growth and even resource exploitation strategies, but how such isolate-specific traits influence competition within host and colonization remains poorly understood. Therefore, our central question is whether more virulent or intrinsically competitive genotypes have a greater probability of dominating within-host competition and determining infection success through host colonization.

Experimental models allow the investigation of how parasite competition drives coinfection outcomes. The fungi *Beauveria* and *Metarhizium* (Ascomycetes: Hypocreales) share many factors in the disease process, but they may differ in ecological traits, taken as competitive abilities, making them interesting for exploring parasite-parasite interactions. These generalist fungi rely on arthropod hosts, such as insects, to reproduce and build population levels (Meyling and Eilenberg 2007). The mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae) is broadly susceptible to generalist fungal infections, and due to its easy mass rearing, small size, and rapid reproduction, is an excellent model for investigating disease. For

example, coinfection of mealworms with *Beauveria* and *Metarhizium* can be directly visualized through post-mortem host colonization, whether by the sporulation of one or both fungi (Figure 1) (Costantin et al. 2025). This characteristic allows us to focus on the parasites' perspective and strategies in the process of host colonization, besides applying to different ecological contexts.

Figure 1. Post-mortem sporulation patterns of *Beauveria bassiana* and *Metarhizium* on the host *Tenebrio molitor* larvae under single and coinfection treatments. (A–B) Single-fungus emergence outcomes in which only one parasite successfully colonized and sporulated on the host cadaver: (A) *B. bassiana* showing characteristic white mycelial growth and conidial coverage, (B) *Metarhizium* showing green conidial sporulation. (C) Coinfected cadaver exhibiting dual sporulation, with both fungi emerging simultaneously from the same host. (D–E) Close-up views highlighting species-specific conidial appearance on the cadaver surface: (D) white conidial sporulation of *B. bassiana*; (E) green conidial sporulation of *Metarhizium.* Photos: the author.

Host colonization can represent the success of infection whether in single or coinfection conditions. However, this success may not be continuous across all coinfecting genotypes as different isolates of the same parasite species differ considerably in virulence. Such variation can play a decisive role in shaping coinfection outcomes, as within-host performance can be highly strain-dependent. This is associated with the genetic background of each isolate, for example, metabolite production, growth rate, and site exploitation. These traits might affect a parasite's ability to compete with other coinfecting parasites and successfully colonize the host. For example, *Metarhizium* can exhibit a diversity of strategies for parasitism and nutrient gain from insects, such as the formation of infection structures, appressoria, and overpowering the host with different enzymes and toxins (Kershaw et al. 1999; St. Leger and Wang 2020; St. Leger 2024).

Parasite isolates can differ markedly in traits such as virulence, growth, and resource exploitation strategies. This intra-specific variation may represent alternative ecological strategies that directly influence infection dynamics and competitive interactions within hosts. Therefore, understanding coinfection outcomes requires explicitly accounting for isolate-level variation as a central component of parasite ecology.

Based on this, we aimed to determine how isolate-specific traits might influence competitive outcomes during coinfection. To address this, we used an experimental system to assess host colonization of ten isolates of *Metarhizium* spp., with different virulence levels, against one *Beauveria bassiana* isolate coinfecting *Tenebrio molitor.* Here, the proportion of host colonization by each isolate in coinfection treatments was taken as a measure of competitive success. We quantified virulence and *in vitro* performance to evaluate how these traits could contribute to within-host dominance. We hypothesized that during coinfection with *Beauveria bassiana*, more virulent isolates of *Metarhizium* spp., or isolates exhibiting more competitive *in vitro* traits, would achieve greater competitive success and colonize a higher proportion of hosts. Our approach provides a parasite-centered perspective on how isolate-level variation could influence competition within-host.

2 MATERIAL AND METHODS

2.1 Fungi

To investigate isolate-specific traits regarding competitive ability on coinfection outcomes, ten isolates of *Metarhizium* spp. were selected to compete against a single *Beauveria bassiana* isolate. The *Beauveria bassiana* isolate used, VIMI-15.0265, was identified as a strong competitor by Costantin et al. (2025) and was used here as a reference for competition comparisons. The isolates were obtained from the Insect-Microorganism Laboratory isolate collection and were originally isolated from soil samples collected in a coffee plantation.

We performed molecular identification of all isolates used in the experiment. For this, we extracted their DNA using the FastDNA SPIN Kit for Plant and Animal Tissue (MP Biomedicals, USA), following the manufacturer's recommended protocol. Cell lysis was performed with BeadBug microtube homogenizer (Benchmark Scientific, UK), allowing the release of DNA without compromising its integrity. We amplified four genomic regions: (i) small nuclear ribosomal subunit (SSU), (ii) large nuclear ribosomal subunit (LSU), (iii) translation elongation factor 1- α (TEF), and (iv) RNA polymerase II largest subunit (RPB1). The primers used for SSU were NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White et al. 1990). Primers for LSU were LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-ATCCTGAGGGAAACTTC-3') (Vilgalys & Sun 1994). Primers for TEF were 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 2218R (5'-ATGACACCRACRGCRACRGTYTG-3') (Rehner & Buckley 2005). Primers for RPB1 were RPB1Ac (5'-GARTGYCCDGGDCAYTTYGG-3') and RPB1Cr (5'-CCNGCDATNTCRTTRTCCATRTA-3') (Murata et al. 2014).

The reactions had a final volume of 25 µL, consisting of 2 µL of extracted fungal DNA, 12.5 µL of Taq polymerase Kapa 2 ReadyMix, 1 µL of each forward and reverse primer [10 pmol/ µL], and 8.5 µL of ultrapure water. The conditions for the amplification of the SSU and LSU regions were as follows: initial denaturation at 95 °C for 3 min; 4 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; followed by 35 cycles of 95 °C for 30 s, 50.5 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 5 min. For the TEF region, the conditions were: initial denaturation at 95 °C for 2 min; 10 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 1 min; followed by 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C

for 1 min; with a final extension at 72 °C for 5 min. For the RPB1 region, conditions were: initial denaturation at 95 °C for 2 min; 10 cycles of 95 °C for 30 s, 64 °C for 1 min, and 72 °C for 1 min; followed by 35 cycles of 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 3 min.

A 1% agarose gel electrophoresis was performed to verify the presence and size of the amplification products. Upon confirmation of sample quality, they were purified and sequenced by Macrogen (Seoul, South Korea). Forward and reverse sequences were conjugated into contigs in Geneious Prime 2025.1.2 (2025). BLAST was used to compare each sequence obtained, to assess its similarities with the GenBank-NCBI database, and identify the species used in this study. The forward and reverse sequences were assembled into contigs. Each sequence obtained was compared using BLAST to assess its similarity with the GenBank-NCBI database in Geneious Prime® 2025.2.1. After BLAST-based identification, we performed multilocus phylogenetic analyses to place the experimental *Metarhizium* isolates in a broader taxonomic context

Phylogenetic analyses were performed using available sequences of *Metarhizium* in NCBI-GenBank, excluding highly divergent and/or overrepresented sequences. After this initial screening and inclusion of our data, the final alignment consisted of 37 sequences (33 SSU, 34 LSU, 19 RPB1, and 30 TEF). We used the species *Metacordyceps owariensis* (MY03260), a phylogenetically close fungal species to *Metarhizium*, as an outgroup. We performed a maximum likelihood (ML) analysis, using ModelFinder and PartitionFinder (Kalyaanamoorthy et al. 2017, Chernormor et al. 2016) to choose the most suitable nucleotide substitution model in the IQ-TREE multicore v. 1.6.12 program. We applied 1,000 Ultrafast Bootstrap replicates (Nguyen et al. 2015; Hoang et al. 2017) and the consensus phylogenetic tree generated was visualized in FigTree v. 3.5.9 and edited in Inkscape (www.inkscape.org). Bootstrap values are shown on each branch.

2.2 Insects

Tenebrio molitor larvae were reared in plastic boxes (21×17×9 cm) with ventilated lids at a room temperature of 25 °C ± 1, under a 12:12 L:D photocycle, and 65 ± 10% relative humidity. The insects were supplied *ad libitum* with a diet of oat bran, wheat fiber, and wheat

bran (in a 1:1:1 ratio). Every three days, slices of carrot or chayote were provided as a supplement. The boxes were checked twice a week and pupated individuals were manually transferred to new plastic boxes containing a paper towel sheet, allowing the emerged adults to hide. After adults emerged, they were placed in plastic containers (41×26×7 cm) with the same diet. Once a month, eggs and newly emerged larvae were collected.

2.3 Coinfection and single infection assay

We performed a coinfection assay to determine the capacities of the *Metarhizium* isolates to dominate hosts in competition with *Beauveria bassiana* and to assess their fitness in this situation. (For simplicity, we refer to these organisms only by genus for the remainder of the chapter.) We also set up single infections of all isolates for comparison and, in all cases, assessed host survival, as virulence (of single or co-infections) might help explain coinfection patterns. For this, fungal suspensions were prepared by flooding selected fungal cultures cultivated on PDA plates with 5 mL of 0.01% Tween 80 and scraping the spores with a sterile Drigalski spatula. 1 mL of each suspension was transferred from the plates into 2 mL Eppendorf tubes. Spore concentrations were determined using a Neubauer Improved chamber and these were adjusted in 0.01% Tween 80 to 1×10^6 spores mL^{-1} for all selected isolates. We tested spore viability by plating an aliquot of 100 µL of each suspension on a PDA culture medium and incubating it at 25 °C for 24 h. Spore suspensions were considered viable if they achieved 95% spore germination; spores were considered to have germinated if their germ tube was longer than the diameter of the spore (Braga et al. 2001). All suspensions used in this experiment showed germination rates above the threshold and were therefore considered suitable for use.

Single and mixed suspensions of *Beauveria and Metarhizium* were prepared as described above. For single infections, 1 mL of each fungal suspension was used. The mixed fungal suspensions were prepared by combining 500 µL of *Beauveria* and 500 µL of *Metarhizium* suspensions at the same final concentration. (Note that this approach of using the same total concentration in coinfections as in single infections, *versus* a double dose, is discussed in Costantin et al. 2025). One microliter of the single or mixed fungal suspensions was inoculated in each *T. molitor* larva (N= 20) per treatment, in the following treatments: 1 – only *Beauveria*; 2 – only *Metarhizium* (for each of the ten isolates); 3 – *Beauveria* + *Metarhizium* (for each of the ten *Metarhizium* isolates); 4- Control (blank 0.01% Tween 80).

As in Costantin et al. (2025), one microliter of the suspension was topically inoculated on the second thoracic segment of *T. molitor* larvae (80-120mg) using a manual pipette. Each insect was observed until the droplet was completely absorbed into the insect's cuticle. In case of droplet loss, through absorption by the filter paper or the insects' natural movement, the larvae were replaced to maintain uniformity regarding the possible reduction of the total dose. After inoculation, each larva was placed in an individualized 15 × 60 mm Petri dish with filter paper moistened with 200 µL sterile water. Mortality was evaluated daily for 15 days; dead insects were placed in an individualized 15 × 60 mm Petri dish humidity chambers with filter paper moistened with 150 µL of sterile water. The plates were sealed with cling film and placed in an incubator (25 +/- 1 °C) for fungal sporulation. The insect cadavers were kept in the humidity chambers for twenty-five days to confirm if the inoculated fungus caused the host's death. After the incubation process, the cadavers were inspected under a stereomicroscope (Olympus SZ61) to assess the percentage of individuals colonized by one or both fungi. *Metarhizium* and *Beauveria* can be distinguished by spore coloration as *Metarhizium* has green spores and *Beauveria* has white spores, making it easy to confirm host colonization by one or both fungi.

To quantify parasite fitness through spore production (based on Costantin et al. 2025), the insect cadavers were placed individually in 2 mL Eppendorf tubes with 1.5 mL of 0.01% Tween 80 solution and vortexed for 1 minute, followed by 10 minutes in an ultrasonic bath to release the spores from the host. Then, the cadavers were kept in the refrigerator at 5 °C overnight. After this, the tubes were centrifuged for 10 min at 14,000 rpm at room temperature, and then vortexed for another minute to resuspend the spores and homogenize the suspension. The spore suspensions were quantified using a cell counting chamber under a microscope. *Metarhizium* and *Beauveria* differ in spore morphology and can be easily distinguished, as *Metarhizium* has a cylindrical aspect and *Beauveria* has a globose aspect. Spore production was estimated for each isolate in single and coinfection to assess each fungal isolate's fitness in the competitive context.

2.4 Conidia germination time

Conidial germination speed was evaluated for the ten *Metarhizium* isolates and the *Beauveria bassiana* isolate, both inoculated separately and together, constituting 21 treatments.

For each treatment, three 90×15 mm Petri dishes containing 1.5% water agar were inoculated with 100 µL of each fungal suspension at 1×10^6 spores mL^{-1}. The plates were kept at 25 °C in the dark. The proportion of germination was assessed for 24 hours by direct observation under a light microscope (Nikon Eclipse E200). For each plate, three arbitrarily selected microscope fields of view were examined at a fixed 40× magnification, and all conidia of each field were counted. Viable germination was defined as described in section 2.3. This procedure allowed us to compare germination kinetics among isolates and relate this to virulence and host colonization performance.

2.5 Fungal performance on artificial media

Fungal performance was assessed on artificial media and on rice, this latter condition was used as a proxy for saprophytic growth - which might correlate with within-host growth and, possibly, competitive ability. Thus, we measured fungal growth and spore yield. All isolates were cultured on 3 different solid artificial media, Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar Yeast extract ¼ (SDAY ¼), and Malt Dextrose Agar, in 90×15mm Petri dishes. Three replicates of each isolate were inoculated on each medium, and the plates were incubated in complete darkness at 25 °C for ten days. Growth was assessed at five and ten days post-inoculation by taking photographs. The images from each evaluation day were used to measure the area of the colony using ImageJ software (Schneider et al. 2012).

Spore production was assessed with fungal growth on rice. For this, 5 g of rice were placed into 50 mL Falcon tubes with 4 mL of water and then sterilized at 120 °C for 15 minutes. After natural cooling, the rice was inoculated with five 5 mm diameter discs from each sporulating isolate that had been cultivated for 15 days on PDA. The inoculated rice was left for ten days in complete darkness at 25 °C. To assess spore production, 15 mL of 0.01% Tween 80 was added to the Falcon tubes and then homogenized to release the spores. The spore suspensions were filtered into 50 mL Falcon tubes using sterile gauze and then diluted for quantification in a cell counting chamber.

2.6 Statistical analyses

Host mortality data were analyzed using Kaplan-Meier survival estimators. The differences among treatments were evaluated with a global Log-Rank test, followed by pairwise comparisons adjusted with Bonferroni corrections ($p < 0.05$). This and the following analyses were run using R software (2024.12.1).

We assessed whether isolate-specific virulence expressed in single infections predicted virulence during coinfection by fitting a linear regression relating mean virulence (1/survival time) in single infections to mean virulence under coinfection.

Host colonization outcomes were analyzed using a multinomial logistic regression model, and the effects of *Metarhizium* isolates on host colonization were tested with a likelihood-ratio test (χ^2). To determine which *Metarhizium* isolate differed from one another, we compared the full multinomial model with level simplifications and compared them with a likelihood-ratio χ^2 test ($p< 0.05$).

We used linear regression analyses with *Metarhizium* isolate as the unit of replication ($n = 10$), using mean values per isolate, to evaluate whether isolate-specific traits predicted infection outcomes. For each regression, we report the regression slope, coefficient of determination (R^2), the F-statistic and associated p-values. Specifically, we tested whether conidial germination speed, quantified as the time required to reach 50% germination (T50), was associated with virulence in single infections and under coinfection with *Beauveria*. We evaluated associations between isolate-specific virulence, quantified as the inverse of host survival time, and infection outcomes during coinfection, including virulence under coinfection, host colonization success, and spore production.

To summarize patterns of *Metarhizium* isolates within-host outcomes during coinfection, a non-metric multidimensional scaling (NMDS) analysis was performed using Bray-Curtis dissimilarities. Ordination was based on variables describing coinfection outcomes, such as the proportion of host colonization and sporulation patterns of each fungus in coinfection treatments. Only numeric variables were used and no data transformation was applied to preserve the biological meaning of the measured traits. To evaluate whether isolate-specific traits were associated with patterns observed in the NMDS ordination, environmental fitting (envfit) was applied using 999 permutations. Continuous traits describing intrinsic isolate characteristics were fitted as vectors onto the ordination space. We included virulence

and spore production on the host under single infection, growth on artificial media, spore production on rice, and germination speed metrics.

3 RESULTS

3.1 Molecular identification of isolates

The isolates of *Metarhizium* belong to three different species: *M. neoanisopliae* (VIMI-10.0022; VIMI-10.0024; VIMI-10.0046; VIMI-10.0115; VIMI-10.0120), *M. humberi* (VIMI-10.0091; VIMI-10.0121) and *M. robertsii* (VIMI-10.0028; VIMI-10.0107; VIMI-10.0125), with *M. neoanisopliae* being the most common (Fig. S1). The tree topology showed that the differences among some isolates are very small, with few substitution sites. In general, there are also isolates in the database that show little variation and require further investigation. Five isolates (VIMI-10.0022; VIMI-10.0024; VIMI-10.0046; VIMI-10.0115; VIMI-10.0120) formed a clade with 64% of support, which is insufficient to support their recognition as a distinct species. The same occurs with isolates that are identified as *M. humberi* (VIMI-10.0091; VIMI-10.0121) and *M. robertsii* (VIMI-10.0028; VIMI-10.0107; VIMI-10.0125).

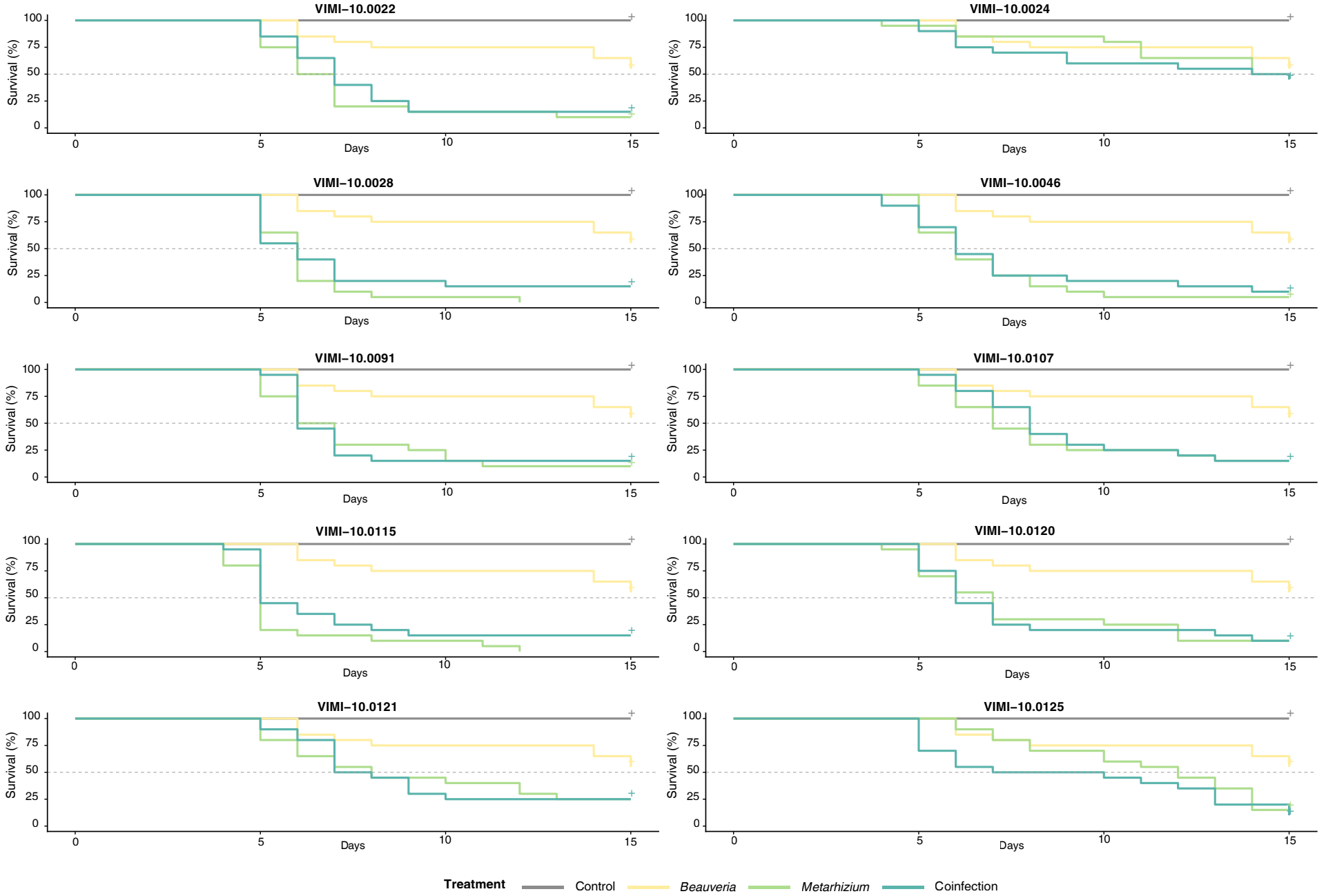
3.2 Coinfection assay

We first quantified host mortality to characterize variation in virulence among *Metarhizium* isolates. Then, we verified if *Metarhizium* virulence expressed in single infections was conserved during coinfection with the competitor *Beauveria,* to determine if differences in host killing were conserved in the presence of a competitor. Additionally, the outcomes of coinfection were quantified in the post-mortem host colonization, considering the possible outcomes: *Metarhizium* or *Beauveria* alone, or both fungi successfully emerging from the same host.

In single infections, we observed substantial isolate-level variation in virulence among *Metarhizium* isolates, expressed as differences in host survival time (Figure 2). Some isolates caused more rapid host death than others, indicating a broad gradient in time to kill the host. Survival curves differed among treatments when analyzed by Kaplan-Meier estimators (global

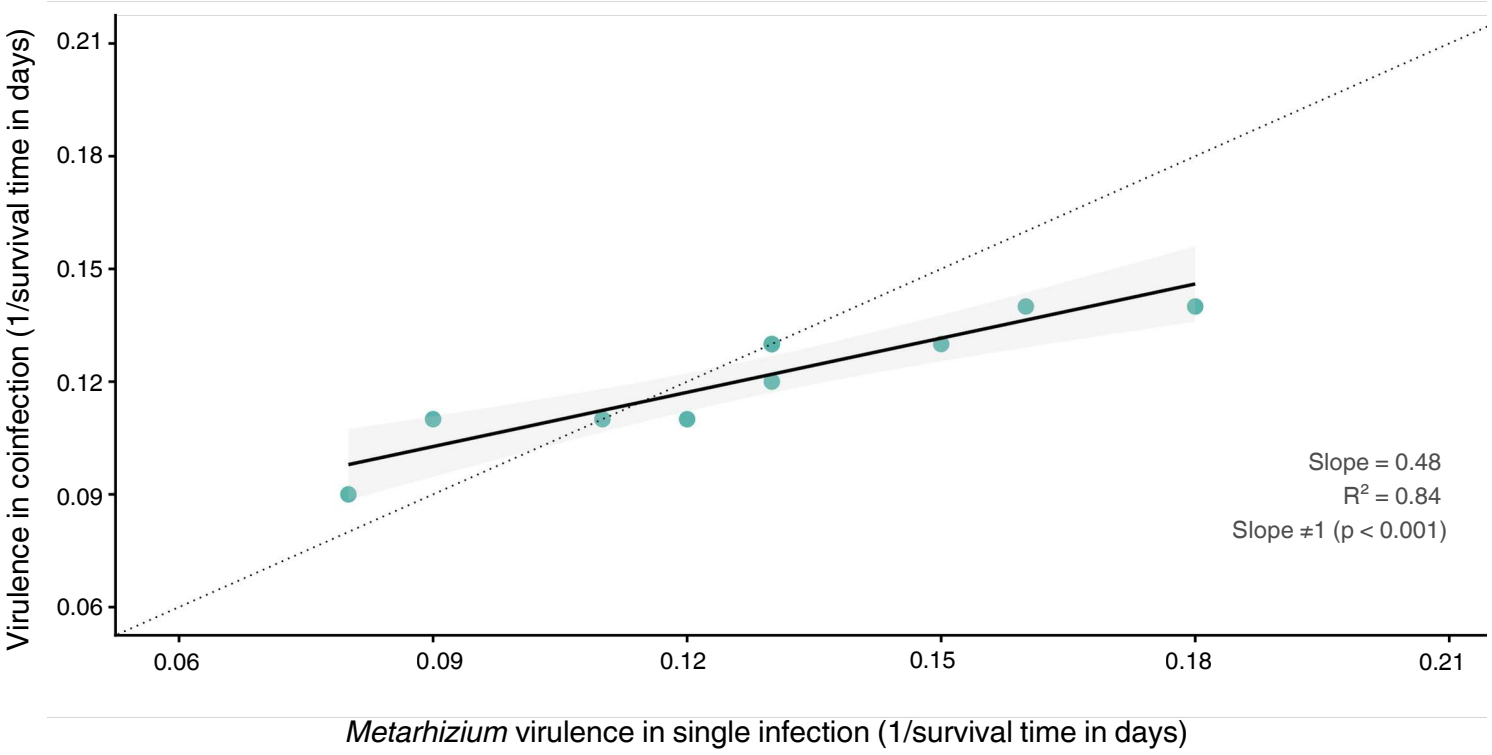
Log-rank test: $\chi^2_{(21)} = 150$, $p < 0.001$), reflecting substantial variation in virulence across *Metarhizium* isolates. Based on median survival times in single infections, isolates VIMI-10.0115, VIMI-10.0028, and VIMI-10.0046 were the most virulent, whereas VIMI-10.0024 and VIMI-10.0125 were among the least virulent. Pairwise comparisons (Log-rank tests with Bonferroni correction) confirmed significant differences between the most and the least virulent isolates, as VIMI-10.0115, VIMI-10.0028, and VIMI-10.0046 differed from VIMI-10.0024 (all $p = 0.001$), and both VIMI-10.0115 and VIMI-10.0028 differed from VIMI-10.0125 ($p = 0.001$). Additional differences were observed, such as between VIMI-10.0046 and VIMI-10.0125 ($p = 0.037$), and between VIMI-10.0121 and VIMI-10.0115 ($p = 0.040$). The *Beauveria* isolate (VIMI-15.0265) occupied the lower end of the virulence gradient and was associated with slower host killing than several *Metarhizium* isolates, differing from six of the ten isolates tested (Log-rank tests with Bonferroni correction: $p = 0.001–0.042$).

Figure 2. Virulence of ten isolates of *Metarhizium* spp. alone or in coinfections with a reference isolate of *Beauveria bassiana* to *Tenebrio molitor* larvae. Shown are Kaplan-Meier survival curves for each *Metarhizium* isolate in single infection and in coinfection with the *Beauveria* isolate (VIMI-15.0265).


VIMI−10.0022
VIMI−10.0024
VIMI−10.0028
VIMI−10.0046
VIMI−10.0091
VIMI−10.0107
VIMI−10.0115
VIMI−10.0120
VIMI−10.0121
VIMI−10.0125
Survival (%)
Days
0
5
10
15
0
25
50
75
100
Treatment
Control
Beauveria
Metarhizium
Coinfection

When *Metarhizium* isolates were tested in coinfection with *Beauveria*, differences in host survival were also observed among isolates. To test whether isolate-specific virulence observed in single infections was conserved under coinfection, we related the *Metarhizium* virulence in single infection to virulence in coinfection using a linear regression model. Virulence in single infections explained 84% of virulence in coinfections ($F_{(1,8)} = 43.32$, $R^2 = 0.84$, $p < 0.001$; Fig. 3). However, the slope test was significantly lower than 1 ($F_{(1,8)} = 50.50$, $p < 0.001$), indicating that virulence in coinfection scaled weakly with virulence in single infections (slope = 0.48).

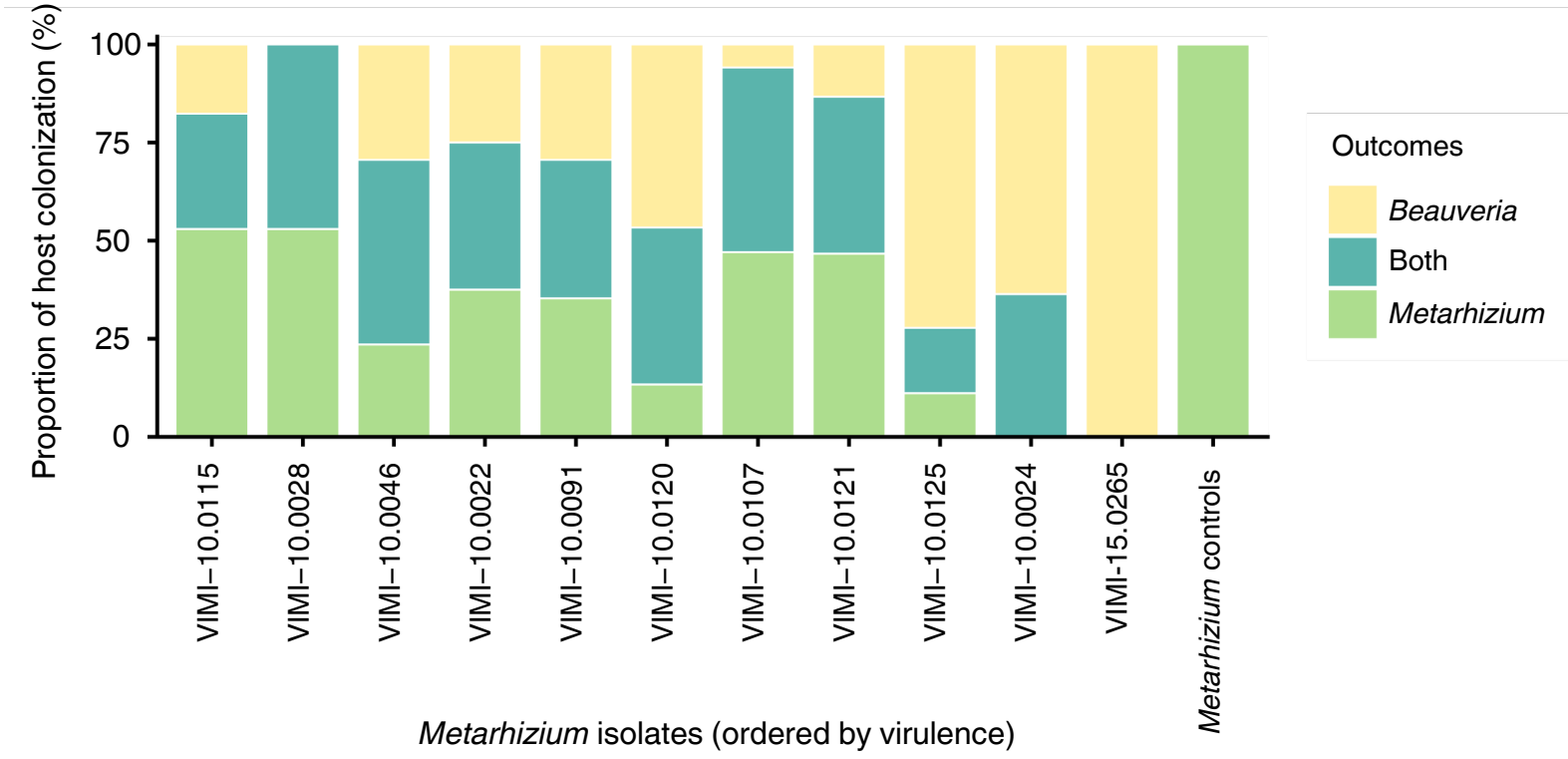
Figure 3. Linear regression model of the relationship between virulence of ten *Metarhizium* isolates in single infections and the virulences when in coinfections with a reference isolate of *Beauveria bassiana* (VIMI-15.0265). Each point represents the mean survival time of *Tenebrio molitor* larvae infected with a given *Metarhizium* isolate in single infection and in coinfection with *Beauveria*.



Substantial variation was observed among *Metarhizium* isolates: ($\chi^2_{(18)} = 52.9$, $p < 0.001$) in the evaluation of host colonization rates – emergence and sporulation (Fig. 4). The isolates VIMI-10.0115 and VIMI-10.0028 showed the highest colonization rates, both reaching 52.9%, whereas VIMI-10.0125 and VIMI-10.0024 showed the lowest colonization rates, with 11.1% and 0%, respectively. The highest proportions of hosts colonized simultaneously by both

fungi were observed in coinfection treatments between VIMI-15.0265 and the isolates VIMI-10.0028, VIMI-10.0046, and VIMI-10.0107, each showing 47.1% of both fungi sporulating.

Figure 4. *Tenebrio molitor* colonization profiles of ten different *Metarhizium* isolates and one *Beauveria bassiana* isolate under coinfection. For each *Metarhizium* isolate, the bars show the proportion of hosts (n= 20) from which *Metarhizium* emerged alone, *B. bassiana* emerged alone, or both fungi emerged from the same host. The bars are ordered from left to right according to the virulence of *Metarhizium* isolates, from highest to lowest (left to right).



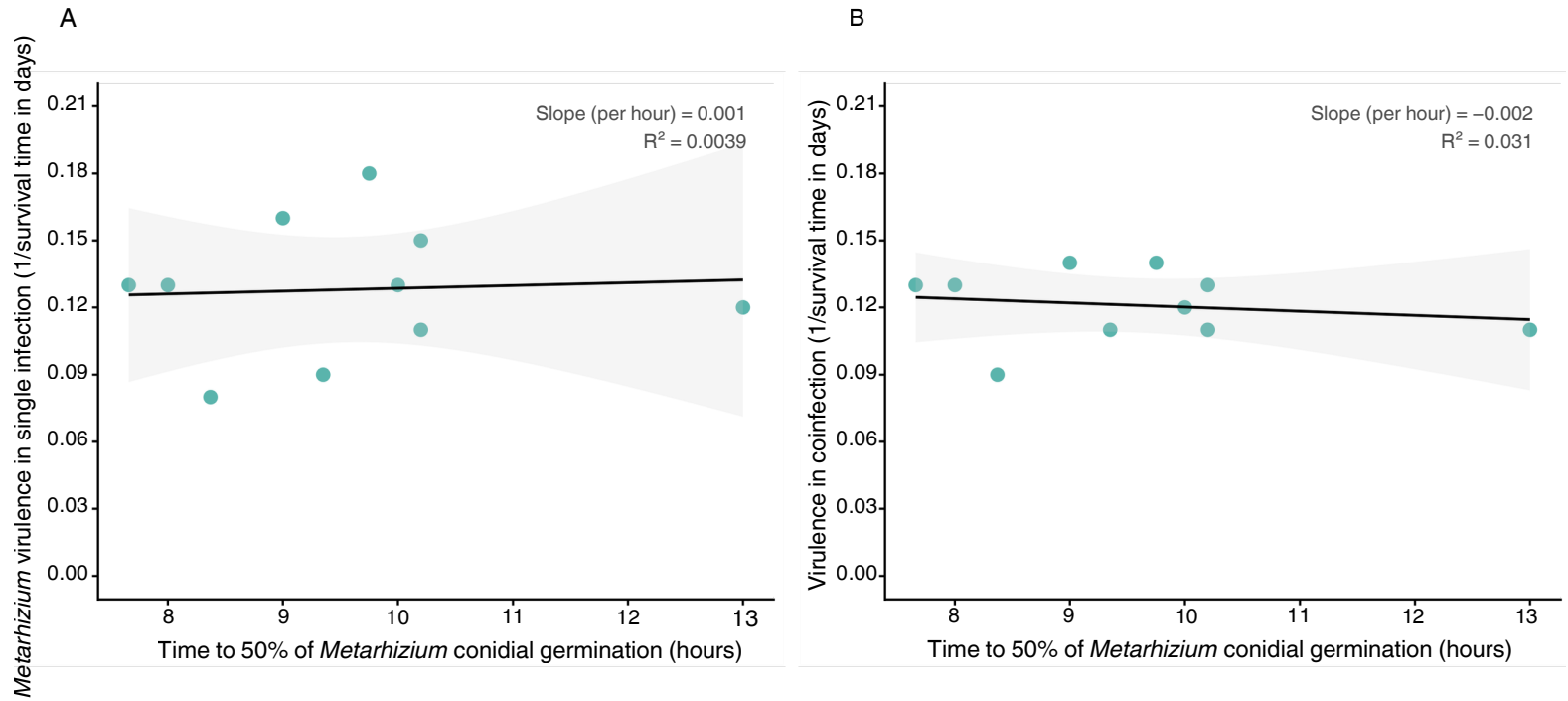
3.3 Virulence–trait associations

We assessed a set of representative traits at different stages of the infection process and related them to virulence, host colonization, and reproductive performance. Traits were selected to capture early infection dynamics, such as conidial germination speed, host killing, through virulence, expressed as 1/host survival time, and host post-mortem resource exploitation, as spore production. Virulence was used as a reference predictor because differences in time to kill the host are expected to influence competitive interactions and access to host resources.

Conidial germination did not contribute to virulence among *Metarhizium* isolates, either in isolation or under competitive coinfection conditions. Specifically, conidial germination

speed was quantified as the time required to reach 50% of germination (T50), and we conducted linear regressions of this with virulences (1/survival time) in single and in coinfections, finding no relationship in either case (Single infections: slope = 0.001 per hour; $F_{(1,8)} = 0.03$, $R^2 = 0.0039$, p = 0.863; Fig. 5A. Coinfections: slope =−0.002 per hour, $R^2 = 0.031$, $F_{(1,8)} = 0.26$, p = 0.0624; Fig. 5B)

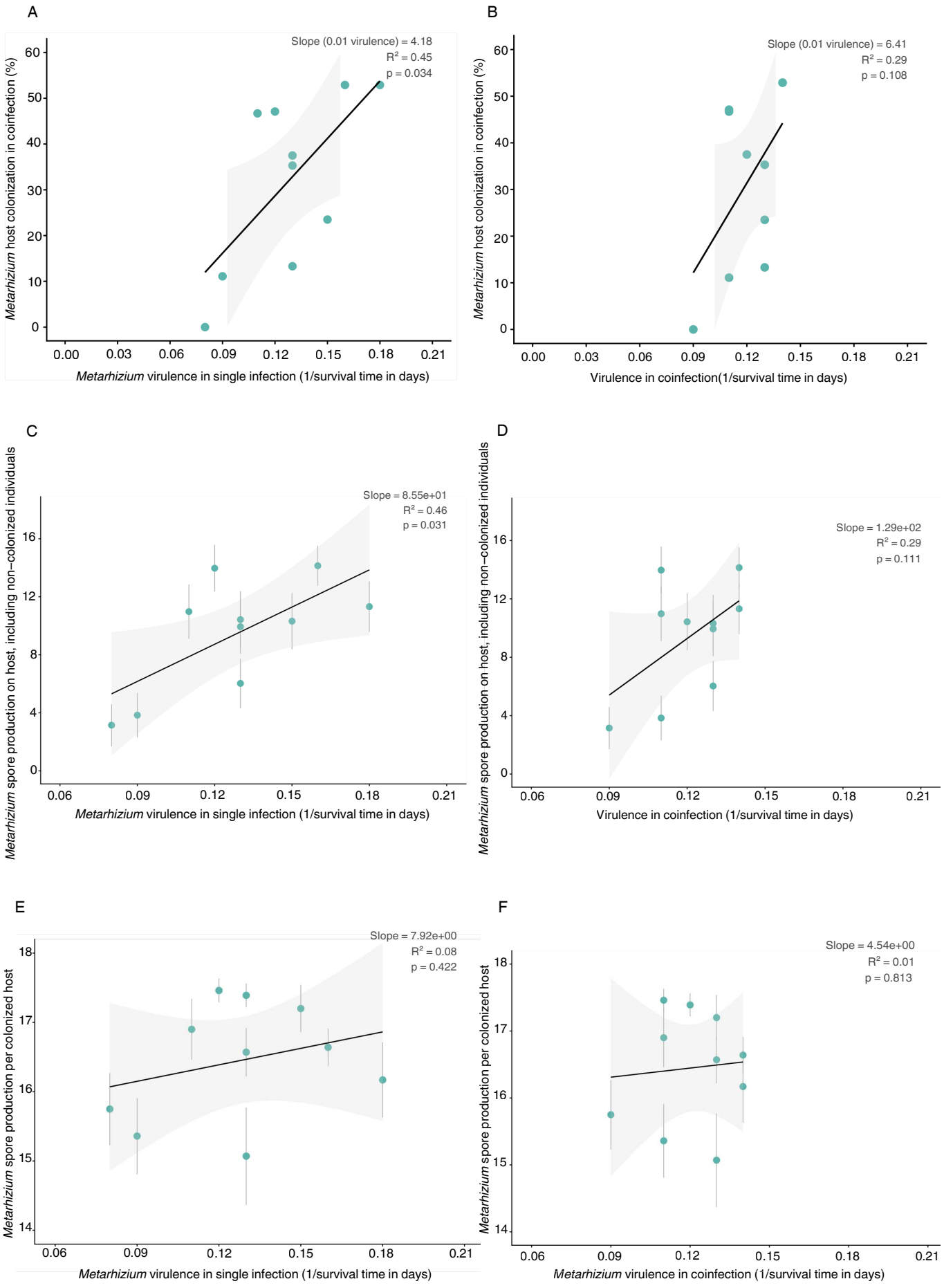
Figure 5. Linear regression between conidial germination speed and virulence of *Metarhizium* isolates in single infection and virulence in coinfection with *Beauveria bassiana.* Scatterplots show linear regressions between the time required to reach 50% conidial germination (T50, hours) and virulence measured as the inverse of host survival time (days). (A) virulence in single infections, (B) virulence under coinfection conditions with *B. bassiana*.



Linear regressions tested whether *Metarhizium* virulence, measured either in single infections or during coinfection, predicts competitive performance during coinfection with *Beauveria* (Figure 6). First, we tested whether virulence predicts host colonization success, quantified as the proportion of hosts (n=20) colonized exclusively by *Metarhizium* after host death in coinfection treatments (Fig. 6A–B). Virulence in the single infection was a positive predictor of colonization success in the coinfection, but this trend was not significant when considering virulence in the coinfection as the explanatory variable (Single infection virulence: slope = 4.18% increase in colonization per 0.01 increase in virulence; $F_{(1,8)} = 6.56$, $R^2 = 0.45$, p = 0.034; Fig. 6A. Coinfection virulence: slope = 6.41% per 0.01 increase in virulence; $F_{(1,8)} = 3.27$, $R^2 = 0.29$, p = 0.108; Fig. 6B).

Considering the population of infected hosts (i.e. all 20 individuals, including those not dominated by *Metarhizium*), virulence expressed in single infections has a positive relation with total spore production ($F_{(1,8)} = 6.78$, $R^2 = 0.46$, $p = 0.031$; Fig. 6C), differently from virulence expressed during coinfection ($F_{(1,8)} = 3.22$, $R^2 = 0.29$, $p = 0.111$; Fig. 6D). It is possible that this relationship could differ when considering only cadavers dominated by *Metarhizium*. This was not the case, however. (Single infection virulence: $F_{(1,8)} = 0.72$, $R^2 = 0.08$, $p = 0.422$; Fig. 6E. Coinfection virulence: $F_{(1,8)} = 0.06$, $R^2 = 0.007$, $p = 0.813$; Fig. 6F).

Figure 6. Relation between isolate-level virulence of *Metarhizium* spp. and competitive outcomes during coinfection with *Beauveria bassiana* (VIMI-15.0265) in *Tenebrio molitor* larvae (n =20). Virulence of *Metarhizium* isolates, measured in single or coinfection, was used as a predictor of three competitive outcomes: the proportion of hosts colonized exclusively by *Metarhizium* in the coinfections, total spore production by *Metarhizium* in hosts colonized by both fungi, and spore production in hosts dominated by *Metarhizium*. (A and B) linear regressions between virulence in single infection (A) or coinfection (B) and the proportion of hosts colonized by *Metarhizium* during coinfection. (C and D) linear regressions between virulence in single infection (C) or coinfection (D) and the spore production by *Metarhizium* per host, including non-colonized individuals. (E and F) show linear regression between virulence in single infection (E) or coinfection (F) and the spore production by *Metarhizium* per colonized host. Each point represents a *Metarhizium* isolate. Regression slopes and coefficients of determination (R^2) are shown within each panel.


A
Slope (0.01 virulence) = 4.18
R² = 0.45
p = 0.034
Metarhizium host colonization in coinfection (%)
Metarhizium virulence in single infection (1/survival time in days)
B
Slope (0.01 virulence) = 6.41
R² = 0.29
p = 0.108
Metarhizium host colonization in coinfection (%)
Virulence in coinfection(1/survival time in days)
C
Slope = 8.55e+01
R² = 0.46
p = 0.031
Metarhizium spore production on host, including non−colonized individuals
Metarhizium virulence in single infection (1/survival time in days)
D
Slope = 1.29e+02
R² = 0.29
p = 0.111
Metarhizium spore production on host, including non−colonized individuals
Virulence in coinfection (1/survival time in days)
E
Slope = 7.92e+00
R² = 0.08
p = 0.422
Metarhizium spore production per colonized host
Metarhizium virulence in single infection (1/survival time in days)
F
Slope = 4.54e+00
R² = 0.01
p = 0.813
Metarhizium spore production per colonized host
Metarhizium virulence in coinfection (1/survival time in days)

3.4 NMDS ordination and isolate-specific trait association

We performed a multivariate analysis to investigate whether coinfection outcomes differed consistently among *Metarhizium* isolates, and whether such patterns could be explained by isolate-specific performance traits.

NMDS ordination was constructed using variables describing the outcomes of coinfection – virulence expressed under coinfection, post-mortem host colonization proportions, and spore production on the host. NMDS organization showed clear differentiation among treatments based on coinfection outcomes, with a well-defined primary axis concentrating the data dominant gradient (stress= 0.036) (Figure 7). The low stress value indicated that the ordination accurately represented the multivariate relationships among treatments. Visually, the ordination suggested that *Metarhizium* isolates differed consistently in their coinfection profiles, supporting the idea of isolate-specific strategies during within-host competition.

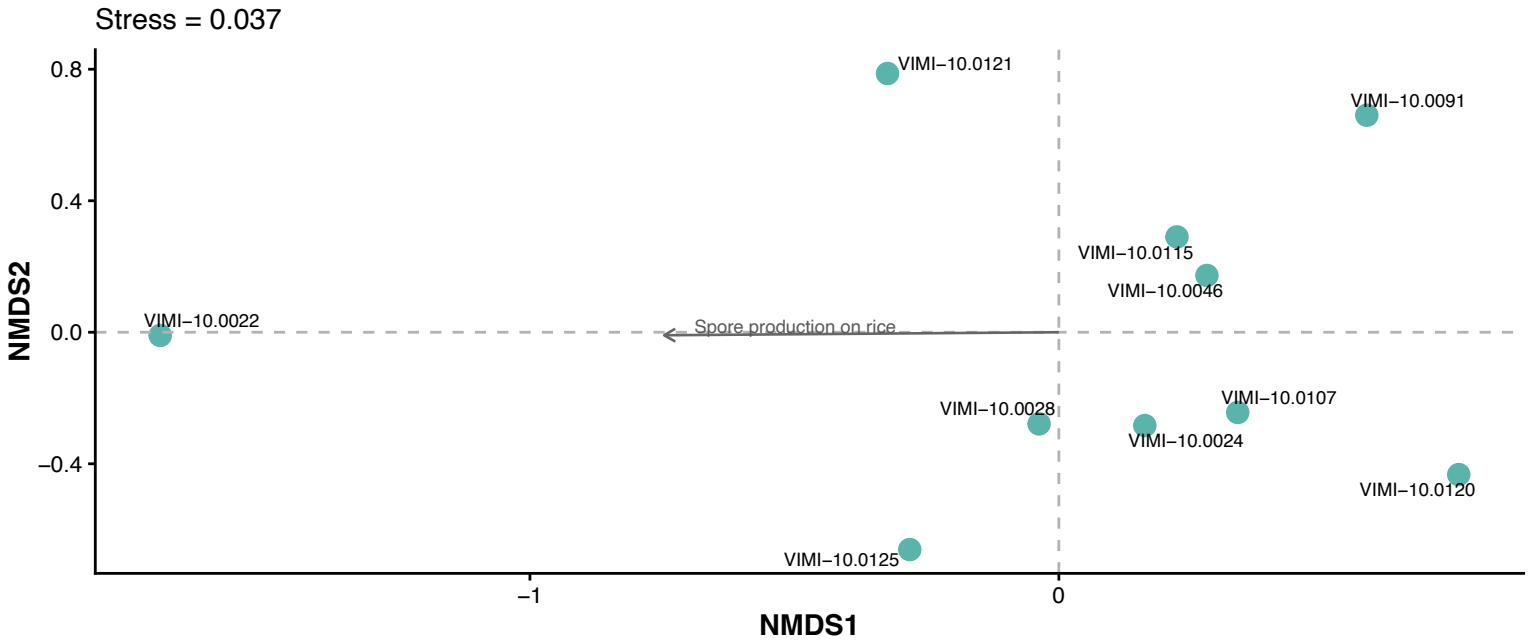


Figure 7. Non-metric multidimensional scaling (NMDS) ordination summarizing coinfection outcomes of multiple *Metarhizium* isolates in interaction with an isolate of *Beauveria bassiana*. The ordination was constructed using Bray–Curtis dissimilarities and resulted in a low stress (stress = 0.037), indicating a robust representation of multivariate patterns. Each point represents a distinct *Metarhizium* isolate positioned according to its coinfection outcome when co-infecting the host with *B. bassiana*. The arrow indicates the fitted vector for saprophytic spore production on rice measured for *Metarhizium* isolates, the only trait significantly associated with the ordination

space (envfit, $r^2 = 0.75$, $p < 0.05$). Vector direction reflects increasing spore production, whereas vector length denotes the strength of the association with the NMDS axes. Other isolate-level traits showed weak or non-significant associations with the ordination and are not shown.

To determine whether isolate-specific performance traits (growth on artificial media, saprophytic performance on rice, germination speed, virulence, and spore production in single infections) could explain this multivariate structure, we tested their association with the NMDS ordination. Among all traits evaluated, saprophytic spore production on rice was the only variable significantly correlated with the ordination ($R^2 = 0.75$, $p = 0.032$). This association closely followed the primary NMDS axis, indicating that variation in saprophytic sporulation was strongly aligned with the dominant gradient structuring coinfection outcomes. All other traits, including virulence and spore production on the host in single-infection treatments, radial growth on different artificial media, and germination speed metrics, showed weak and non-significant associations with the ordination space. Although these traits vary among isolates, they do not consistently align with the multivariate structure of *Metarhizium* isolate competitive patterns in the coinfection outcomes.

4 DISCUSSION

Coinfections represent a realistic ecological context in which parasite fitness is determined by host exploitation and by interactions among competing parasites. In this study, we investigated how isolate-level variation within *Metarhizium* affects competitive outcomes during coinfection with *Beauveria* in the model host *Tenebrio molitor*. We integrated survival analyses, host colonization patterns, reproduction metrics, and multivariate ordination approaches in a parasite-centered framework.

Phylogeny confirmed that the ten isolates used in this study represent taxonomic diversity within *Metarhizium*, being assigned to three species: *M. neoanisopliae*, *M. robertsii*, and *M. humberi*. Despite the low phylogenetic divergence among several isolates, competitive outcomes during coinfection differed markedly and were strongly predicted by isolate-level virulence. These results highlight that phenotypic traits relevant to infection success and competition are unlikely to be explained by species identity alone. Instead, variation in

virulence and within-host competitive outcomes can plausibly arise from intraspecific diversity and isolate-specific genetic mechanisms

The survival analyses revealed well-defined differences in virulence among *Metarhizium* isolates, highlighting substantial isolate-level variation in their ability to kill the host (Fig. 2). Single infection survival curves differed across isolates, as some isolates caused rapid host mortality, whereas others exhibited prolonged host survival. This virulence variation observed among *Metarhizium* isolates is likely associated with differences in their genetic background, reflecting the high intraspecific diversity of this genus. Thus, it was expected that isolate-specific infection strategies would show different patterns of host mortality and exploitation. In this context, virulence should be viewed not as a single trait but as the outcome of multiple genetic processes, for example, metabolite expression and growth dynamics. This provides a mechanistic basis for the heterogeneity observed in virulence outcomes and why isolates occupying similar ecological niches display contrasting virulence profiles.

When we compared *Metarhizium* single infections to coinfections with *Beauveria* at the same inoculum concentrations, we did not observe a synergistic increase in virulence in these treatments, such as that found by Rao et al. (2006), Leal-Bertioli et al. (2000), and Wang et al. (2002). In some *Metarhizium* isolates, coinfection resulted in survival patterns like those observed in single infections, consistent with Costantin et al. (2025) and Pauli et al. (2018), who also observed that coinfection did not consistently accelerate host death relative to single infections. Nevertheless, coinfection effects were not consistent across isolates, indicating that the impact of *Beauveria* on host mortality depends on the identity of the competing *Metarhizium* isolate.

Early infection traits can influence host mortality; for example, the variation in conidial germination speed can contribute to isolate-specific differences in virulence (Faria et al. 2015). Conidial germination represents a critical early step in the infection process of entomopathogenic fungi, however, the time required to reach 50% of germination did not predict virulence among *Metarhizium* isolates (Fig. 5). Although germination competence is important to the infection process, subsequent stages of infection play a more dominant role in determining host survival. Cuticle penetration efficiency, within-host proliferation, immune evasion, and toxin production are likely to have a stronger impact on host mortality. The differences observed in the germination speed variation reflect the differences in fungal physiology that are not directly linked to pathogenic competence. Our findings support that competitive interactions with *Beauveria* do not increase the importance of early germination

dynamics. Additionally, virulence in obligate-killing entomopathogens might be better explained by processes occurring after host invasion.

The strong positive relationship between *Metarhizium* virulence in single infection and virulence during coinfection treatments demonstrates that isolate-specific differences in virulence are largely conserved even in the presence of a competing parasite (Fig. 3). However, the slope of 0.48 indicates that although isolates maintained consistency in virulence across contexts, differences among isolates were reduced under coinfection. It suggests that the presence of *Beauveria* attenuated isolate-level variation in virulence, consistent with both competitor effects and the reduced per-species dose of *Metarhizium* in the combined inoculation.

Then, we assessed these coinfection outcomes considering three possible results regarding host colonization: only *Metarhizium,* only *Beauveria*, or both fungi colonizing the same host simultaneously (Fig. 4). We observed a high heterogeneity among *Metarhizium* isolates in their ability to colonize hosts during coinfection. Some isolates consistently achieved higher proportions of host colonization, either alone or in combination with *Beauveria*, whereas others were largely excluded. The frequent occurrence of dual sporulation indicates that competition between both fungi was not strictly exclusionary, as both fungi persisted within the host, whether through spatial partitioning of host tissues or different growth rates.

Host colonization was expected to increase with isolate virulence, as higher virulence would confer advantages in coinfections by allowing faster host exploitation, better resource use, and higher rates of host colonization by that isolate. This provided an important complement to virulence and colonization results by revealing a quantitative link between *Metarhizium* virulence in single infection treatments and competitive success in coinfection (Fig. 6A). In this condition, approximately half of the variation in host colonization by *Metarhizium* during coinfection was explained by differences in virulence, suggesting that it plays a meaningful but not exclusive role in shaping the outcomes in this study system. Li et al. (2021) also observed that in the *Beauveria-Metarhizium-Tenebrio* system, the *Metarhizium* isolate tested was a dominant competitor as it killed the host faster than *Beauveria*. These results are consistent with the idea that more virulent isolates gain an early temporal advantage during infection, probably by rapidly breaching host defenses and proliferating in the hemocoel. This temporal advantage may represent an earlier access to host tissues and surface colonization, increasing the likelihood of dominating the cadaver during post-mortem sporulation.

However, several isolates deviated substantially from the regression line, indicating that isolates with similar virulence can differ markedly in colonization success. It also suggests that a rapid host killing alone is insufficient to guarantee dominance in a competitive scenario, as traits acting after host death or those related to resource acquisition or saprophytic growth could play a more decisive role in determining coinfection outcomes.

The spore production by *Metarhizium*, quantified at the level of the host population, including non-colonized individuals, showed no relationship with *Metarhizium* virulence in single infections or coinfections (Fig. 6C & D). This was also the case when we considered *Metarhizium* spore production and these virulences (Fig. 6E & F). It indicates that isolate-specific differences in reproductive capacity after successful host colonization are largely independent of virulence expressed during infection. Both results suggest that once *Metarhizium* successfully colonizes the host, variation in spore yield is not related to how rapidly the host can be killed. Instead, reproductive performance could be governed by traits expressed during post-mortem growth, such as saprophytic capacity, which may vary independently of virulence.

As virulence indicated a context-dependent trait in which fitness consequences can differ substantially between single and coinfections, our approach was then to investigate whether other isolate-level traits could influence competition more than virulence alone. In this context, the NMDS ordination provided an integrated view of coinfection outcomes by summarizing multiple variables (Table S1) into a multivariate space. The low stress value indicated that the ordination captured the structure of the data, allowing meaningful biological interpretation of the relative positions of *Metarhizium* isolates. The clear separation among isolates in the ordination space indicates consistent and isolate-specific coinfection profiles. To interpret this structure, we applied environmental fitting (envfit) using isolate-specific traits such as growth in different artificial media, spore production on rice, and spore germination speed metrics into the NMDS ordination (Table S2). It revealed that spore production on rice was the only variable significantly aligned with the ordination space. Although rice is an artificial substrate, this metric is widely used as a proxy for fungal performance under saprophytic growth conditions, as it simulates natural environmental conditions (Miranda-Hernándes et al. 2017; Patiño-Medina et al. 2021).

Following host death, entomopathogenic fungi undergo an extended phase of post-mortem growth and sporulation, during which the insect cadaver effectively functions as a saprophytic substrate. The strong association between spore production on rice and the NMDS

structure suggests that isolates with superior saprophytic performance are more efficient at exploiting host cadavers and, consequently, more likely to dominate or persist during competition in coinfections. This pattern supports the idea that traits related to resource exploitation, rather than host killing per se, are central to competitive interactions between entomopathogenic fungi.

When we compared the regression analyses with the NMDS ordination and envfit analysis, virulence explained a substantial proportion of host colonization in univariate analyses, yet failed to structure the multivariate coinfection space as strongly as saprophytic spore production on rice. As host death is not the endpoint of the infection for entomopathogenic fungi, these fungi rely on the host cadaver as a substrate for reproduction, unlike obligate parasites whose fitness is maximized through host survival or transmission before host death. Consequently, selection may act less strongly on killing speed and more on traits expressed after host death, such as saprophytic growth.

Although this system allowed precise control of host and competitor identity, some limitations should be acknowledged. The coinfections were performed using a single *Beauveria bassiana* isolate under constant laboratory conditions, which simplifies the competitive scenario relative to abiotic conditions. However, this controlled design was intentional once we focused on isolating specific variation within *Metarhizium* isolates to be the primary source of variation in competitive outcomes.

Our results suggest an order of processes shaping competitive outcomes, in which virulence determines early access to the host, while post-mortem traits determine reproductive dominance. Although virulence explained a substantial portion of the competitive success of *Metarhizium* during coinfection with *Beauveria*, our results revealed that virulence alone does not determine within-host dominance. However, traits associated with post-mortem exploitation of the host, particularly saprophytic sporulation capacity, emerged as a central determinant structuring the multivariate pattern of host colonization. These findings support the hypothesis that, for entomopathogenic fungi, fitness is not restricted to the speed of host death but is strongly linked to the efficiency with which the host cadaver is exploited as a resource for reproduction and persistence. We demonstrated that intraspecific variation could generate contrasting competitive strategies within a coinfection system, and this work highlights the importance of considering multiple functional dimensions to explain interactions among microorganisms within hosts.

5 SUPPLEMENTARY MATERIAL

Figure S1: Phylogeny of *Metarhizium* obtained using Maximum likelihood analysis with 1,000 bootstraps of the concatenated alignment of the four genomic regions LSU, SSU, TEF and RPB1. Isolates from this study are in bold. *Metacordyceps owariensis* MY03260 was used as outgroup.

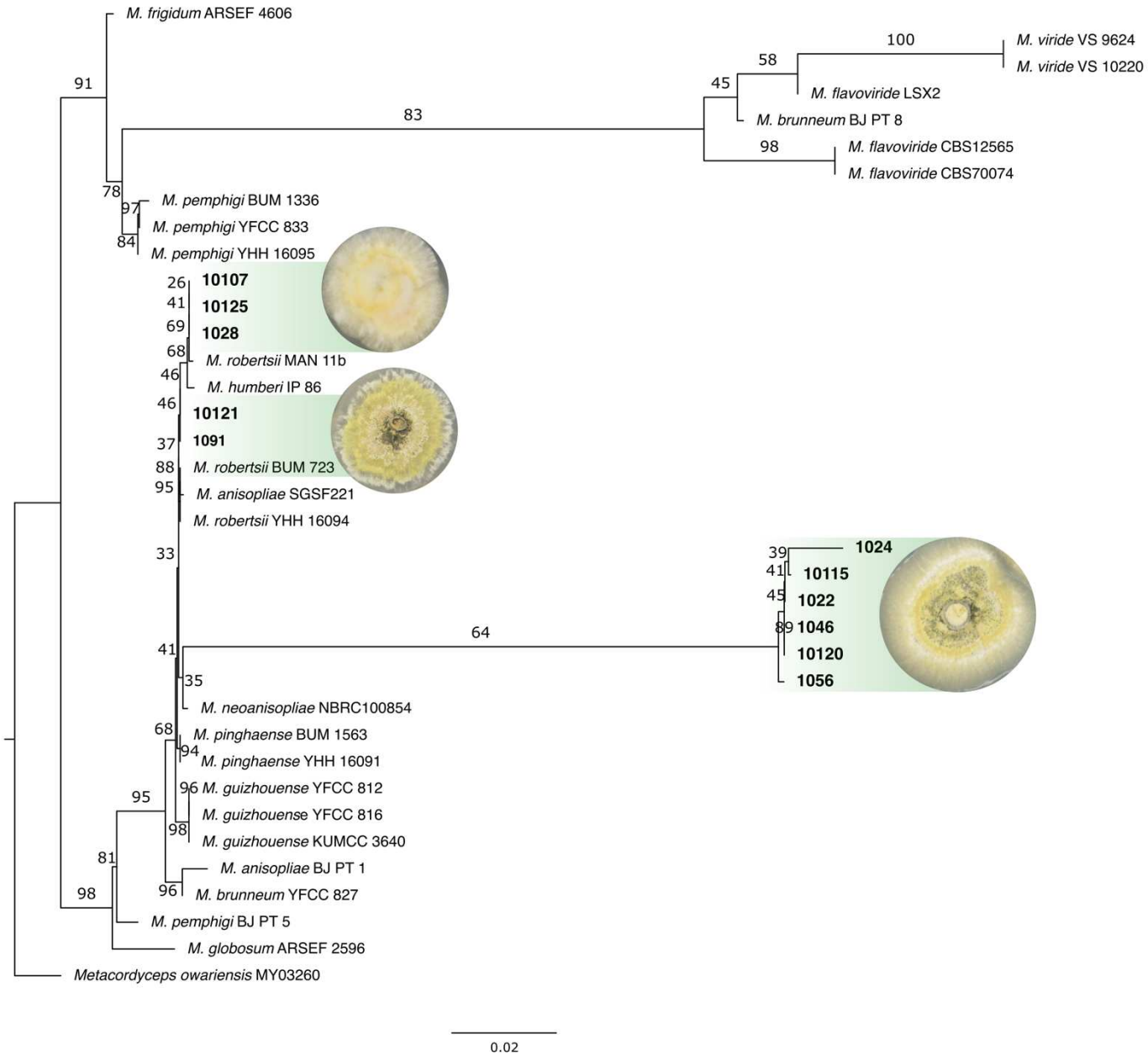


Table S1. Summary data used to build the NMDS ordination for the coinfection treatments. For each *Metarhizium* isolate coinfected with *Beauveria bassiana*, the table reports treatment means for virulence in coinfection, proportion of post-mortem colonization, and spore production in coinfection for each fungus.

	Virulence	Host colonization			Spore yield on host (Coinfection)	
Isolate	Coinfection	*Metarhizium*	*Beauveria*	Coinfection	*Metarhizium*	*Beauveria*
VIMI-10.0022	8,05	0,375	0,250	0,375	31109375	3787500
VIMI-10.0024	11,45	0,000	0,636	0,363	3454545,45	13863636,4
VIMI-10.0028	7,3	0,529	0,000	0,47	24073529,4	1088235,29
VIMI-10.0046	7,55	0,235	0,294	0,47	32823529,4	3970588,24
VIMI-10.0091	7,65	0,352	0,294	0,352	15897058,8	6882352,94
VIMI-10.0107	9,1	0,470	0,058	0,47	43058823,5	1794117,65
VIMI-10.0115	7,1	0,529	0,176	0,294	24838235,3	4647058,82
VIMI-10.0120	7,7	0,133	0,466	0,4	7616666,67	12206666,7
VIMI-10.0121	9,2	0,466	0,133	0,4	34695000	2448333,33
VIMI-10.0125	9,35	0,111	0,722	0,166	2161764,71	16500000

Table S2. Envfit results for the NMDS ordination, using treatment means of *Metarhizium*-specific variables only to identify which traits drive the NMDS structure (r^2 and permutation p-values).

	Virulence	Mean spore yield on rice	Mean spore yield on host (single infection)	Growth in artificial media			Time to 50% of conidial germination	
Isolate	*Metarhizium*			PDA	SDAY	Malt	*Metarhizium* alone	*Metarhizium* coinfection
VIMI-10.0022	7,4	273333333333333	564073529411765	14,19	6,13	5,07	13,00	10,00
VIMI-10.0024	12	39400000	7117500	11,53	7,02	4,57	11,30	8,37
VIMI-10.0028	6	166666667	27363750	11,06	3,54	4,91	9,66	9,00
VIMI-10.0046	6,8	304000000	446302631578947	13,98	9,70	10,26	11,70	10,20
VIMI-10.0091	7,5	115666667	795277777777778	17,21	6,20	10,88	10,60	8,00
VIMI-10.0107	8,5	2915000000	109264705882353	10,94	6,42	8,10	12,50	13,00
VIMI-10.0115	5,6	790666667	57175000	12,00	7,62	10,53	9,80	9,75
VIMI-10.0120	7,9	614000000	528666666666667	9,40	9,93	13,94	7,43	7,66
VIMI-10.0121	9,5	813333333	50306250	8,38	8,89	15,65	10,60	10,20
VIMI-10.0125	11	2532666667	143019230769231	11,66	6,68	14,80	11,40	9,35

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COMPARATIVE HISTOPATHOLOGY OF *METARHIZIUM–BEAUVERIA* COINFECTIONS IN *TENEBRIO MOLITOR*

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ABSTRACT

Coinfections are common in natural populations and can strongly influence parasite fitness by modifying within-host competition and host exploitation. We conducted a comparative histopathological assessment of single and coinfections of the mealworm *Tenebrio molitor* by the entomopathogenic fungi *Metarhizium* and *Beauveria*. Two isolates of *Metarhizium neoanisopliae* previously categorized as high- and low-virulence were coinfected with a *Beauveria* isolate to characterize within-host distribution, tissue tropism, fungal morphology, and host cellular responses. Larvae were fixed immediately after death and longitudinal sections were stained with hematoxylin and eosin. Across treatments, fungal structures were most consistently associated with the fat body, whereas muscle tissue was comparatively preserved. In single infections, *Beauveria* showed readily detectable fungal structures and widespread tissue association, whereas fungal structures were limited or not readily detectable in the low-virulence *Metarhizium* isolate. In contrast, the high-virulence *Metarhizium* isolate displayed more extensive tissue occupation, including fungal structures within muscle tissue, relative to the low-virulence isolate. Coinfections consistently showed fungal structures and hemocyte presence at tissue interfaces, suggesting concurrent fungal development and host cellular responses. Together, these observations link isolate-level virulence with contrasting within-host exploitation patterns and provide qualitative support for competition-driven variation in fungal development within hosts.

Keywords: within-host interactions, host exploitation, entomopathogenic fungi, tissue tropism, fungal virulence

1 INTRODUCTION

When parasites share the same mode of establishment and within-host niche, the possible outcomes of mixed infections can be shaped by their intrinsic traits and interactions they hold (Sheng and St. Leger 2024). In this scenario, parasite species share resources and face the host's immune defenses, forcing them into competition either for nutrients (exploitative competition) or through immune-mediated interactions (apparent competition) (Ramesh and Hall 2025). These complex within-host processes can lead to diverse optimal strategies for coping with competitors. For example, when host resources are limiting, a parasite's faster exploitation strategy should outperform more prudent ones.

In insects, multiple fungal infections typically result in competitive rather than synergistic interactions (Li et al. 2021). In this case, fungi interfere with each other's growth and establishment by altering the host environment and competing for space and nutrients. Entomopathogenic fungi are therefore a valuable model system for investigating coinfections aspects as they frequently co-occur in nature, infecting the same host, and competitive traits can be experimentally quantified.

Our goal was to describe histological parameters of coinfection and investigate whether competition for space and resources, aligned with differences in virulence, could influence fungal performance and development within the host at the tissue level. For this, we used an experimental model comprised two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium* spp. (*Acomycetes: Hypocreales*), coinfecting the host *Tenebrio molitor* (Coleoptera: Tenebrionidae), as developed by Costantin et al. (2025). This system enables a parasite-centered perspective on within-host interactions. Although *Beauveria* and *Metarhizium* can share many factors regarding the disease process, they may differ in ecological traits and competitive abilities, including at isolate level, providing an opportunity to focus on parasite ecology. On the other side, *T. molitor* is highly susceptible to fungal infections and provides a robust model to study pathological aspects.

Pathogenicity begins when fungal conidia attach to the insect cuticle, and penetrate it through a combination of turgor pressure, mechanical force and cuticle-degrading enzymes (Ortiz-Urquiza and Keyhani 2013; Shang et al. 2024). Once inside the host environment, selection favors traits that maximize growth (Coombs et al. 2007). Fungi propagate as blastospores or hyphal bodies (St. Leger and Wang 2020), acquiring nutrients and evading

inflammatory cells. Progressive tissue disruption from hyphal invasion and nutrient depletion kills the host (Gul et al. 2014), after which the fungus grows saprophytically.

The histological study of infections is a primary source for assessing pathogen distribution and tissue alterations (Lee et al. 2021). In our study system, we aimed to identify and describe the histology of fungal establishment, hyphal morphology, and density during *Metarhizium* and *Beauveria* single and coinfection in *T. molitor*. We additionally compared two *Metarhizium* isolates representing contrasting virulence levels (high vs low), to test whether variation in virulence could be associated with differences in host establishment and exploitation under single and coinfection contexts.

This chapter presents as an explanatory histopathological assessment, which describes tissue-level infection patterns in single and coinfections. The observations provide a qualitative base for subsequent quantitative analyses.

2 MATERIAL AND METHODS

2.1 Insects

Adults of *Tenebrio molitor* were maintained in plastic containers (41×26×7 cm) and supplied *ad libitum* with a diet of oat bran, wheat fiber, and wheat bran (in a 1:1:1 ratio). Every month, eggs and newly emerged larvae were collected and transferred to plastic boxes (21×17×9 cm) with ventilated lids and kept at a room temperature of 25 ± 1 °C, under a 12:12 L:D photocycle, and 65 ± 10% relative humidity. During this phase, larvae were supplied *ad libitum* with the same diet, and every three days, slices of carrot or chayote were provided as a supplement. The boxes were checked twice a week, and pupated individuals were manually transferred to new plastic boxes containing a paper towel sheet, allowing the emerged adults to hide.

2.2 Fungi and infection

To investigate possible correlations between virulence, growth and competitive abilities, we selected two *Metarhizium neoanisopliae* isolates representing contrasting virulence levels: a high-virulence isolate (VIMI-10.0115; hereafter *Metarhizium*-high) and one low-virulence isolate (VIMI-10.0024; hereafter *Metarhizium*-low) (see Chapter 2), plus a reference *Beauveria bassiana* isolate, VIMI-15.0265, to assess their within-host growth in single and coinfections. The isolates were recovered from the Insect-Microorganism Laboratory isolate bank.

For *Beauveria* and *Metarhizium* isolates, we prepared fungal suspensions by flooding selected fungal cultures cultivated on PDA plates with 5 mL of 0.01% Tween 80 and scraping the spores with a sterile Drigalski spatula. Then, 1 mL of each suspension was transferred from the plates into 2 mL Eppendorf tubes. The spore concentrations were determined using a Neubauer Improved chamber and these were adjusted in 0.01% Tween 80 to 1×10^6 spores mL^{-1} for all selected isolates. The viability of suspensions was tested by plating an aliquot of 100 µL of each suspension on a PDA culture medium and incubating it at 25 °C for 24 h. It was considered viable if 95% of spore germination was achieved (Braga et al. 2001). All suspensions used in this study were considered suitable for use.

For single infections, 1 mL of each fungal suspension was used. The mixed fungal suspensions were prepared combining 500 µL of *Beauveria* and 500 µL of *Metarhizium* suspensions at the same final concentration. We had the following six treatments: *Metarhizium*-high, *Metarhizium*-low and *Beauveria* for single infections, and *Metarhizium*-high with *Beauveria*, and *Metarhizium*-low with *Beauveria*, for coinfections, plus a control group.

Host infection was performed by topically inoculating 1 µL of the single or mixed fungal suspensions on the second thoracic segment of *T. molitor* larvae (100-120 mg) using a manual pipette - as in Costantin et al. (2025). The insects were observed individually until the droplet was completely absorbed. If the droplet was lost the larvae were replaced to maintain uniformity and avoid possible reduction of total dose. Then, the larvae were placed in individualized 15 × 60 mm Petri dishes with filter paper moistened with 200 µL sterile water. The mortality was assessed daily until larvae death.

2.3 Histology

We performed the histopathological analyses of *Metarhizium-Beauveria* coinfection on the host *T. molitor* to evaluate morphological aspects of fungal infection, growth and development, and host tissue exploitation. To achieve this, three larvae per treatment (n= 3) were infected as described in section 2.2. Immediately after death, larvae were immersed in ten times their volume of 10% buffered formalin (pH 6.8) as a fixative to preserve host tissue and fungal structures at a standardized post-mortem time point. To achieve better results in fixative infiltration, we sectioned each larva transversely.

The samples were dehydrated in increasing ethanol series, 80%, 90%, 100% I, 100% II, 100% III, and embedded in liquid paraffin to support cutting sections. After paraffin polymerization, the samples were cut into 5 µm-thick sections using a microtome. The sections were submitted to conventional hematoxylin-eosin (HE) staining. Uninfected larvae were equally processed for comparative purposes. The histological samples were analyzed and photographed under a light microscope.

Here, histological analyses were conducted primarily to describe within-host distribution of fungal structures. *Quantitative image-based measures - fungal area coverage, hemocyte counts, and hyphal morphometrics, are currently being developed and will be incorporated in the final version of this paper.*

3 RESULTS

Based on the virulence ranking obtained in Chapter 2 (Kaplan–Meier survival analyses), we selected two *Metarhizium* isolates representing contrasting levels of virulence (high and low virulence) to investigate if within-host development and tissue exploitation differed at the histopathological level and their possible interactions with a *Beauveria* isolate. As larvae were fixed immediately after death, sections represent comparable post-mortem conditions, although treatments differed in time to death

3.1 Infection distribution, tissue-level patterns of host exploitation and fungal morphology of *Metarhizium*-low

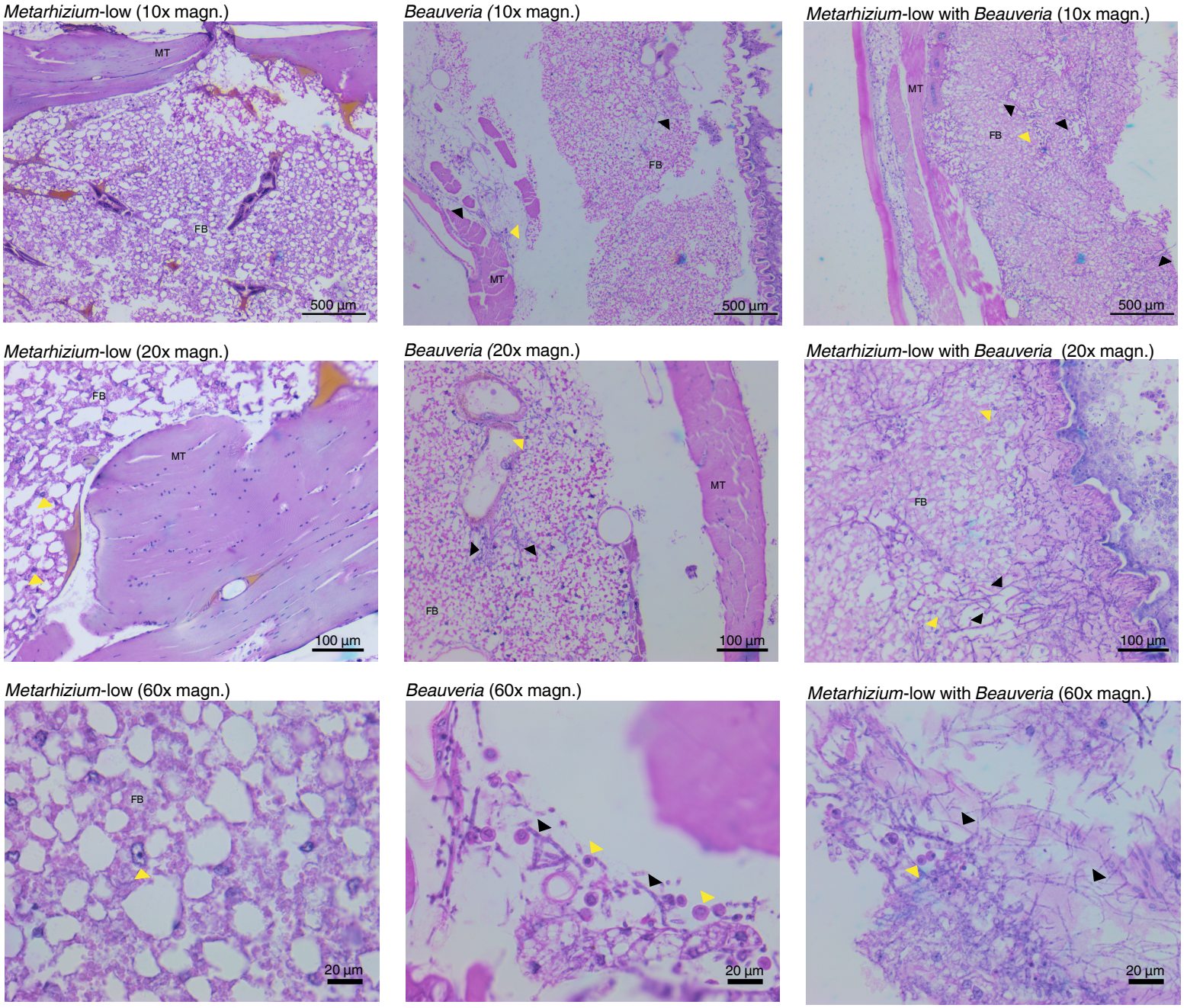

Figure 1. Comparative histopathology of single and coinfections in *Tenebrio molitor* larvae using *Metarhizium*-low isolate (VIMI-10.0024) and *Beauveria* (VIMI-15.0265). Longitudinal histological sections stained with hematoxylin and eosin are organized by treatment and magnification. Larvae were fixed immediately after death. The fat body (FB) and muscle tissue (MT) are indicated. Black arrowheads indicate fungal structures within host tissues, while yellow arrowheads indicate hemocytes (host immune cells) in the host tissue.

During the infection with *Metarhizium*-low in single infection, fungal presence was not easily detectable in the examined sections and in different magnifications, with apparent limited tissue occupation. In contrast, *Beauveria* in single infection showed clear fungal presence with multiple regions exhibiting fungal structures consistent with systemic infection development (Figure 1). Fungal structures were detectable in *Metarhizium*-low coinfecting the host with *Beauveria*, indicating successful infection development under coinfection. Notably, hemocytes were present in the hemocoel, even when fungal structures were not prominent in the *Metarhizium*-low single infection.

At the tissue level, fungal structures were predominantly detected in association with the fat body (FB), whereas muscle tissue (MT) was generally more preserved in treatments with limited fungal tissue occupation. In the *Metarhizium*-low single infection, no fungal structures were detected in the examined sections, either in FB or in MT. In this treatment, hemocytes were observed in association with the fat body, whereas fungal structures were not observed in the examined sections.

In contrast, in *Beauveria* single infections, fungal structures were easily observed, primarily associated with the fat body, while muscle tissues retained their organized fiber structure and appeared preserved. In the coinfection involving *Metarhizium*-low with *Beauveria*, fungal structures were evident and again most consistently associated with the fat body, whereas muscle tissue remained relatively preserved in this treatment.

At high magnification, the morphology of fungal structures differed qualitatively between *Beauveria* and *Metarhizium* treatments. In *Beauveria* infections, fungal structures generally appeared as relatively thin hyphal structures.

3.2 Infection distribution, tissue-level patterns of host exploitation and fungal morphology of *Metarhizium*-high

In the single infection of *Metarhizium*-high, fungal structures were more widely distributed across the section than in *Beauveria*, as total infection appeared to show more extensive tissue occupation (Figure 2). Fungal presence was also consistently detected in the *Metarhizium*-high with *Beauveria* coinfection treatment, as fungal structures were widely distributed throughout the sections, with frequent detection in both fat body and muscle tissues.

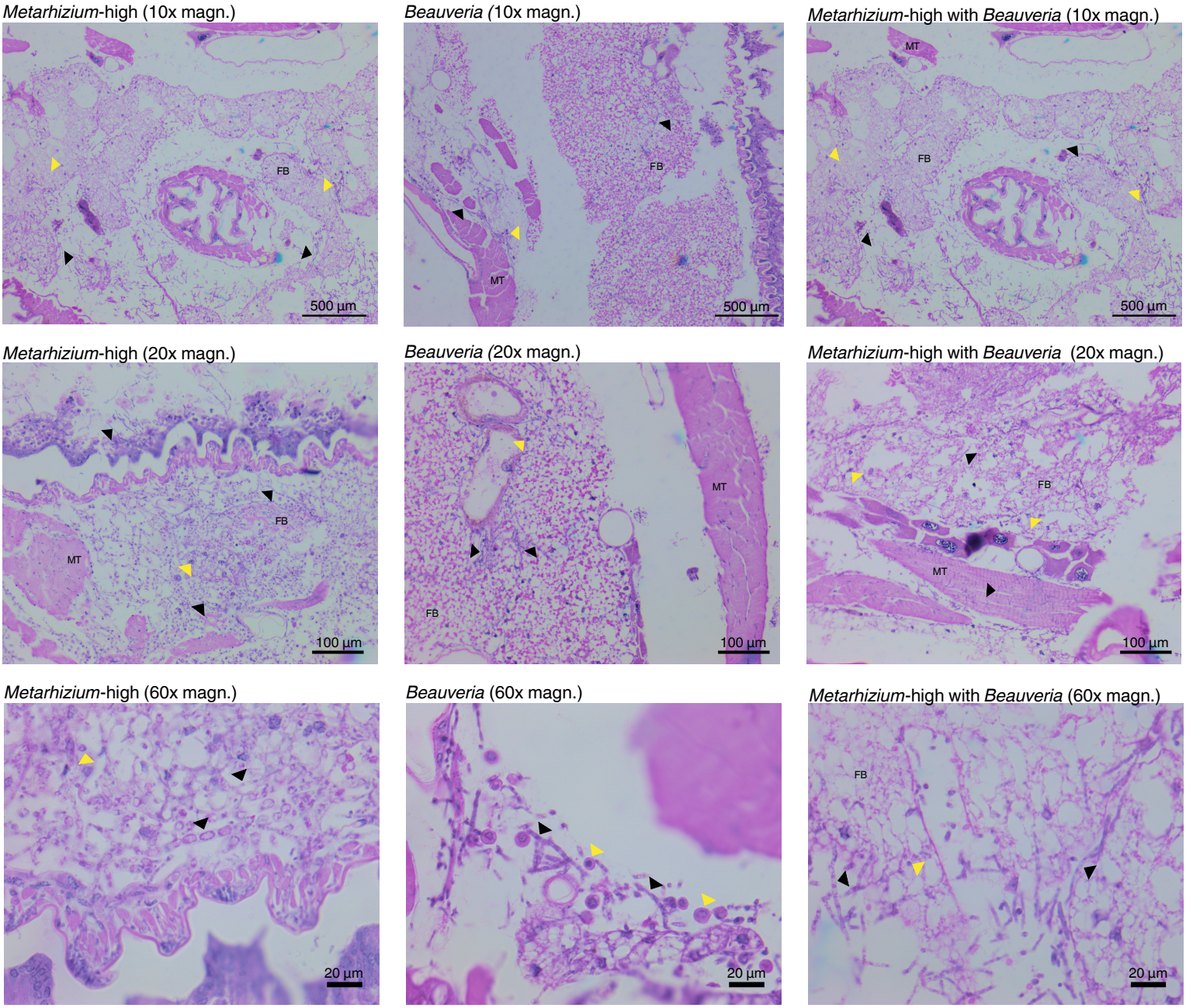

Figure 2. Comparative histopathology of single and coinfections in *Tenebrio molitor* larvae using *Metarhizium*-high isolate (VIMI-10.0115) and *Beauveria* isolate (VIMI-15.0265). Longitudinal histological sections stained with hematoxylin and eosin are arranged by treatment and magnification. Larvae were fixed immediately after death. Tissue regions corresponding to the fat body (FB) and muscle tissue (MT) are labeled. Black arrowheads indicate fungal structures and tissue occupation, whereas yellow arrowheads indicate hemocytes (host immune cells), highlighting host cellular responses associated with infection.

Tissue-level patterns differed markedly in treatments involving the *Metarhizium*-high isolate, as fungal structures were widely present and strongly associated with the fat body, with a more extensive tissue occupation than observed in the *Metarhizium*-low isolate. In addition, fungal structures were also observed within muscle tissue, consistent with invasion of MT. In the coinfection, *Metarhizium*-high with *Beauveria*, fungal structures were widespread, with intense occupation of the fat body and muscle tissue, which appeared markedly affected by fungal growth.

In contrast, in *Metarhizium*-high infections, fungal structures appeared more robust, with hyphae showing a thicker aspect relative to those observed in *Beauveria*. In coinfection sections, fungal structures with both thin and robust hyphal profiles were observed.

Overall, histological observations showed clear differences in within-host fungal development between the *Metarhizium* isolates categorized as low- and high-virulence in Chapter 2. Fungal structures were not detected in the *Metarhizium*-low single infection, whereas they were widely distributed in larvae infected with the *Metarhizium*-high isolate. Across treatments, fungal structures were most consistently detected in association with the fat body, while muscle tissue was comparatively preserved except in treatments involving the *Metarhizium*-high isolate, even in coinfections. Hemocytes were frequently observed in the tissues, even in treatments involving the *Metarhizium*-low isolate - where no fungal structures were observed.

4 DISCUSSION

By comparing longitudinal sections of *Tenebrio molitor* larvae infected with *Beauveria* and two *Metarhizium* isolates representing contrasting virulence levels, we identified consistent tissue-level patterns of fungal presence and host cellular responses in single and coinfections. Across treatments, infection was primarily associated with the fat body, while muscle tissue was comparatively preserved in most cases. However, the *Metarhizium*-high isolate showed more extensive tissue occupation and evidence of fungal structures in muscle tissue, in contrast to the *Metarhizium*-low isolate, linking isolate-level virulence differences with contrasting within-host exploitation.

A key aspect of our results is that larvae were fixed immediately after death, ensuring that observed fungal structures reflect infection status at a standardized post-mortem period and with minimal saprophytic growth. The larvae did die at different moments among treatments, consistent with the marked differences in virulence (see Chapter 2). This temporal difference requires a careful interpretation as a more virulent isolate causes earlier death and may establish more quickly within the host, whereas less virulent isolates may reflect a slower proliferation, or a stronger host resistance. Therefore, differences in tissue occupation between treatments may be associated with differences in infection rate and host exploitation capacity. The absence of detectable fungal structures in *Metarhizium*-low single infections, combined with hemocyte presence, suggests that host defenses may have either limited fungal proliferation and delay systemic development, or potentially eliminated the infection. In contrast, the extensive tissue occupation observed in *Metarhizium*-high indicates rapid progression to generalized infection, consistent with the isolate's virulence ranking. In this direction, the histology complements that a possible high virulence is not only an outcome in time to host death but also may correspond to a distinct within-host phenotype.

After penetrating the integument and entering the hemocoel, infecting fungus can undergo a dimorphic transition, from hyphal growth to the production of free-floating single-celled hyphal bodies (Zhang et al. 2024). These cells can travel through the host open circulatory system, infecting internal tissues, as fat body (Toledo et al. 2010).

The histopathological study is particularly informative for evaluating this spatial within-host distribution, host tissue exploitation, and evidence of host cellular responses. In our system, hematoxylin and eosin (HE) staining allowed clear identification of major tissue compartments (fat body, hemocoel, muscle tissues) and detection of fungal structures, as hyphae, enabling relevant comparisons between treatments. This approach is especially valuable in entomopathogenic fungi, where infection success depends also on within-host proliferation and post-mortem exploitation.

At the same time, HE has limitations regarding parasite identity in coinfections. While *Beauveria* and *Metarhizium* can display qualitative differences in hyphal appearance, definitive attribution of fungal structures to species is challenging without targeted staining or other techniques. For this reason, the coinfection interpretations here are best framed in terms of fungal presence, tissue occupation, and hypha morphology. However, the qualitative differences in fungal abundance and distribution between the low- and high-virulence *Metarhizium* treatments strongly supports isolate-specific differences in within-host dynamics.

Across all treatments, fungal structures were most consistently detected in association with the fat body (Butt et al. 2001). This pattern is biologically expected given the central role of the insect fat body as a nutrient-rich and metabolically active tissue. This region is a major site for lipid storage and protein metabolism. For pathogens, it represents a resource reservoir that can sustain rapid growth once systemic invasion has occurred. Colonization of fat body may represent a strategy of converting host metabolic stores into fungal tissues. The consistent detection of hemocytes in the fat body also suggests that the host immune system is active at this site (Kamimura et al. 2020), which may be a determinant of isolate-level differences in infection success.

A notable difference in the *Metarhizium*-high isolate was the evidence of more extensive tissue occupation, including muscle tissues. Muscle is typically less colonized early in infection relative to other tissues (Toledo et al. 2010), and its disruption suggests an advanced systemic stage. This could be explained as higher virulence may reflect accelerated proliferation within-host, enabling the fungus to reach higher densities and invade a wider range of tissues, including muscle tissues. Also, isolate-specific traits may increase the capacity for tissue penetration and invasion, potentially involving stronger production of tissue-degrading compounds and secondary metabolites (Gibson et al. 2014; Hu et al. 2016; Zhang et al. 2024).

Across both isolate combinations, coinfections consistently showed fungal structures and hemocytes, indicating that coinfections result in host cellular responses. This aligns with the broader ecological framework developed in Chapter 1 and the competitive outcomes measured in Chapter 2: within-host competition does not eliminate infection establishment, but it might change the trajectory of resource exploitation and the spatial development of infection.

In the coinfection involving the *Metarhizium*-low isolate, the fungal structures observed in this coinfection may primarily reflect *Beauveria* growth, which would be consistent with *Beauveria* being a strong competitor and reliable colonizer. In contrast, the coinfection involving the *Metarhizium*-high isolate showed extensive tissue occupation, including involvement of muscle, indicating that the aggressive within-host trajectory of this isolate could be maintained in the presence of a competitor.

Although the present chapter focuses on qualitative histological comparisons, this approach is intended as a first step toward a quantitative framework. By integrating histopathology with the isolate-level virulence framework established in Chapter 2, our findings provide mechanistic support for the idea that *Metarhizium* isolates differ in within-host

infection capacities. The fat body emerges as the primary site of fungal presence and likely represents the main resource niche exploited during infection. The *Metarhizium*-high isolate exhibited a more aggressive infection phenotype characterized by widespread tissue occupation, whereas the *Metarhizium*-low isolate showed limited detectable fungal development in single infection but evidence of host cellular response. Coinfections consistently displayed systemic fungal presence and hemocyte accumulation, indicating that coinfections involve both fungal development and host immune responses. Together, these observations reinforce the importance of considering isolate-level traits to understand the outcomes of fungal coinfections in insect hosts.

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