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Thermal ecology shapes disease outcomes of entomopathogenic fungi infecting warm-adapted insects

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ABSTRACT

The thermal environment is a critical determinant of outcomes in host-pathogen interactions, yet the complexities of this relationship remain underexplored in many ecological systems. We examined the Thermal Mismatch Hypothesis (TMH) by measuring phenotypic variation in individual thermal performance profiles using a model system of two species of entomopathogenic fungi (EPF) that differ in their ecological niche, *Metarhizium brunneum* and *M. flavoviride*, and a warm-adapted model host, the mealworm *Tenebrio molitor*. We conducted experiments across ecologically relevant temperatures to determine the thermal performance curves for growth and virulence, measured as % survival, identify critical thresholds for these measures, and elucidate interactive host-pathogen effects. Both EPF species and the host exhibited a shared growth optima at 28 °C, while the host's growth response was moderated in sublethal pathogen infections that depended on fungus identity and temperature. However, variances in virulence patterns were different between pathogens. The fungus *M. brunneum* exhibited a broader optimal temperature range (23–28 °C) for virulence than *M. flavoviride*, which displayed a multiphasic virulence-temperature relationship with distinct peaks at 18 and 28 °C. Contrary to predictions of the TMH, both EPF displayed peak virulence at the host's optimal temperature (28 °C). The thermal profile for *M. brunneum* aligned more closely with that of *T. molitor* than that for *M. flavoviride*. Moreover, the individual thermal profile of *M. flavoviride* closely paralleled its virulence thermal profile, whereas the virulence thermal profile of *M. brunneum* did not track with its individual thermal performance. This suggests an indirect, midrange (23 °C) effect, where *M. brunneum* virulence exceeded growth. These findings suggest that the evolutionary histories and ecological adaptations of these EPF species have produced distinct thermal niches during the host interaction. This study contributes to our understanding of thermal ecology in host-pathogen interactions, underpinning the ecological and evolutionary factors that shape infection outcomes in entomopathogenic fungi. The study has ecological implications for insect population dynamics in the face of a changing climate, as well as practically for the use of these organisms in biological control.

1. Introduction

Temperature plays a crucial role in host-pathogen interactions and disease dynamics in natural systems, particularly for ectothermic species (Rohr & Cohen, 2020). Body temperature significantly influences the performance and fitness of the interacting species, and taking temperature into account is therefore vital for understanding the ecology and evolution of host-pathogen interactions (Martin & Huey, 2008). For example, infection with a pathogen can affect the host's capacity to respond to thermal stress (Hector et al., 2021), which may have important implications for pathogen transmission as the climate warms, including a greater likelihood of disease outbreaks (Elderd & Reilly, 2014).

Even when the thermal sensitivities of both host and pathogen align, the smaller size of pathogens compared to the host has led to the assumption that pathogens often have a broader range of optimal temperatures than hosts (Rohr & Cohen, 2020). However, individual performance across thermal gradients for hosts and pathogens is not always aligned and even small changes in environmental temperature can significantly affect host traits and the outcome of host pathogen

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interactions. The thermal mismatch hypothesis (TMH) accounts for such cases where host and pathogen fitness peak at different temperatures. The TMH asserts an increased susceptibility of hosts to pathogens under environmental conditions that diverge from their ideal thermal range (Cohen et al., 2020, Rohr & Cohen, 2020). That is, warm-adapted hosts are more susceptible to pathogen infection at colder temperatures, and cold-adapted hosts are more susceptible at warmer temperatures.

There is broad support for the TMH across wildlife populations, with the most extensive evidence coming from amphibian systems (Cohen et al., 2020). A significant effect of thermal mismatch has been observed for fungal pathogens infecting warm-adapted hosts at cold temperatures, where fungal pathogenicity increases as temperature drops (Cohen et al., 2020). This pattern of thermal mismatch is, for example, evident between amphibian hosts from colder or warmer climatic conditions and the fungal pathogen *Batrachochytrium dendrobatidis*, which leads to temperature-dependent host susceptibility to fungal disease (Cohen et al., 2017). However, this outcome is not universal for fungal pathogens (see for example, Sun et al., 2023), and it is challenging to predict the effect of warmer or colder temperatures on a particular host-fungal interaction.

Many of the host and pathogenic fungal traits that play crucial roles in determining infection outcomes are highly influenced by temperature, and insect-pathogenic fungi specifically are sensitive to microclimatic variations because they infect insects directly through the host cuticle (Cory et al., 2010). Temperature also influences key aspects of the interaction itself, such as host resistance, immune response and recovery, in addition to pathogen virulence, activation and replication (Herren *et al.*, 2023, Hector et al., 2023). These effects are not always linear, as they arise from complex "genotype-by-genotype-by-environment" interactions. This implies that distinct thermal responses for various traits are the result of the interplay of genetic characteristics of both host and parasite, leading to a diverse range of responses that can variably impact both host survival and parasite virulence (Thomas and Blanford, 2003; Hector et al., 2023; Sinclair et al., 2022; Gehman et al., 2017). Furthermore, temperature may have indirect effects on the host and pathogen that are not fully understood. For example, infection indirectly affects host responses to temperature (Hector et al., 2021), while the growth response of the host to changes in temperature may be critical for pathogen transmission because host growth affects the production of pathogen propagules, such as fungal spores (Boomsma et al., 2014). In experimental studies, the relationships between temperature and virulence are usually represented as a thermal virulence performance curve, which captures the dynamic interaction between the host and pathogen across different temperatures (Thomas and Blanford, 2003). Most pathogenic fungi do not perform well at warm temperatures, which, despite notable exceptions, helps to reduce risk of fungal infection for warm-blooded mammals (Robert & Casadevall, 2009). This has been proposed as a selective pressure in the evolution of endothermy, aiding in the prevention of fungal infection for warm-blooded mammals (Casadevall, 2012). However, ectothermic organisms such as insects, amphibians, reptiles, and plants may be more susceptible to fungal infection, which is also reflected in recent fungal epidemics discovered in amphibians and plants (Casadevall, 2005; Fisher & Denning, 2023; Fisher et al., 2022; Fisher et al., 2020; Fisher et al., 2012; Fones, Fisher & Gurr, 2017; Harvell et al., 2002).

The ability to infect insects has evolved multiple times within the kingdom fungi, and today entomopathogenic fungi are globally widespread and present wherever insects are found (Humber, 2008). Entomopathogenic fungi exhibit diverse ecologies ranging from obligate and host-specific to facultative or opportunistic pathogens with a broad host-range, and includes some with extensive parts of their lifecycles occurring in the soil or as plant symbionts (Boomsma et al., 2014; Cory et al., 2010). In the latter group, the entomopathogenic fungal genus *Metarhizium* has evolved from root symbiotic fungi colonizing plant roots, and is primarily found in the soil environment (Sheng et al., 2022; Stone & Bidochka, 2020; Vega and Kaya, 2012). The genus *Metarhizium* is globally distributed, inhabiting a range of habitats across tropical and temperate soils where these fungi play an important role in regulating natural insect populations (St Leger & Wang, 2020; Roy et al., 2009). There is wide variation in fungal performance at different temperatures among *Metarhizium* species, in part correlating with geographical origin, resulting in significant isolate-specific temperature performance curves (Chandra Teja & Rahman, 2016; Ouedraogo et al., 1997; Driver et al., 2000). However, the effect of fluctuating or constant temperatures on *Metarhizum* spp. growth does not always correlate with virulence in their insect host (Meissle et al., 2023). Acclimation and thermal history of both insect host and *Metarhizium* fungus can contribute to the outcome of infection at different thermal conditions (Ferguson & Sinclair, 2020). In general, many fungi perform best at cool to intermediate temperatures (Carlile et al., 2001), which suggests that entomopathogenic fungi may have a particular advantage when infecting warm-adapted hosts at cold temperatures.

A thermal performance curve describes how an organism performs for a given trait (growth, virulence, etc.) across a range of temperatures, whereas the thermal niche is the range of temperatures within which an organism can survive, grow, and reproduce (Gvoždík, 2018). For pathogenic fungi, the thermal niche also delineates the temperatures at which they can effectively infect hosts. The thermal niche is a component of the infective niche (Rogers et al., 2013), which is defined as the suite of conditions, including temperature, that permits a pathogen to infect and cause disease in a host. In the present study, we aimed to quantify the phenotypic variation in thermal performance curves of two entomopathogenic fungal species differing in their ecological niche and evolutionary histories, and assess the thermal performance curve of the outcome of the interaction between entomopathogenic fungi and a warm-adapted insect host. In particular, we asked: (1) Do individual pathogens and hosts exhibit overlapping or distinct thermal performance curves and optima, and (2) How do host growth and pathogen virulence outcomes reflect the interaction between the host and pathogen's individual thermal performance curve.

To answer these questions, we compared the ecological differences between species in the entomopathogenic genus *Metarhizium* that differ in their evolutionary history. We used *M. brunneum* and *M. flavoviride* as pathogens in virulence tests with a model insect host, larvae of the mealworm *Tenebrio molitor*, which were warm acclimated to 28 °C and where temperature is known to influence the immune response (Catalán et al., 2012). Both species of fungi are common soil fungi in Northern Europe but vary in their global abundances and virulence (Keyser et al., 2015; St Leger & Wang, 2020; Sheng et al., 2022). *Metarhizium flavoviride* is locally abundant in Denmark, but is often reported as infrequently isolated in other parts of the world (Fisher et al., 2011; Keyser et al., 2015; Sun et al., 2008; Tóthné Bogdányi et al., 2019; Wyrebek et al., 2011). The *Metarhizium* genus contains species with broad host ranges and high global abundance. For example *M. brunneum*, part of the *Metarhizium anisopliae* complex (Mongkolsamrit et al., 2020), has a wide host range, in part due to an ancestral horizontal gene transfer of genes responsible for cuticle-degrading enzymes (Zhang et al., 2019), and is among some of the most isolated *Metarhizium* species from soil globally (St Leger & Wang, 2020). The wide host range and frequent isolation of *M. brunneum* from soil is in concordance with a broad ecological and infective niche (Meyling & Eilenberg, 2007). Conversely, *M. flavoviride*, in the *M. flavoviride* complex (Mongkolsamrit et al., 2020), exhibits a narrower infective niche, targeting fewer insect host species (Moonjely & Bidochka, 2019; Keyser et al., 2016). Many isolates of *M. flavoviride*, previously designated *M. flavoviride* var. *flavoviride*, are known to be cold-active, germinating and growing at low temperatures (<10 °C) (Driver et al., 2000). These distinct evolutionary histories and ecological adaptations may therefore have led to differences in their thermal and infective niches, and we expect *M. flavoviride* to be more cold-adapted than *M. brunneum*. Predictions of the TMH thus suggest that *M. flavoviride* will have higher virulence towards warm-adapted *T. molitor* at colder temperatures, but differences in ecological niches

and adaptive traits of the two pathogens will also affect their virulence and growth at various temperatures.

2. Materials and methods

2.1. Insect strain and fungal isolates

A laboratory culture of *T. molitor* maintained at 28 °C for at least 10 generations was used in experiments. Insects were kept in plastic boxes (750 ml volume with dimensions of 15 cm × width 9.5 cm × depth 4 cm) at 28 °C in the dark and provided with a diet comprising wheat bran, supplemented with 5 % w/w egg white powder (Millipore® Merk). Insects were fed small plugs of 1 % w/v water agar (Bacteriological (European Type) No. 1, Neogen Corp.) twice a week to provide a source of water.

Two species of *Metarhizium* spp. isolated in Denmark were used in this study, namely *M. brunneum* KVL 12_30 and *M. flavoviride* KVL 14_112 (Steinwender et al., 2014; Keyser et al., 2016), both of which were isolated from the soil using *T. molitor* larvae as a bait insect (Steinwender et al., 2014). The acronym KVL refers to the entomopathogenic fungus culture collection maintained at the Section for Organismal Biology, Department of Plant and Environmental Sciences, University of Copenhagen.

2.2. Preparation of fungal inoculum

The inoculum for experiments was prepared from in vitro cultures of each of the fungal isolates. Fungal cultures (3 replicate plates) of each isolate were grown on quarter- strength Sabouraud dextrose agar + yeast media (SDAY/4: 2.5 g/L 1:1 animal: bacterial peptone (Bacteriological Peptone & Acuferm Neoeptone, Neogen Corp.), 10 g/L dextrose (Bacteriological, Oxoid Ltd.), 2.5 g/L yeast extract (Neogen Corp.), 15 g/L agar (Bacteriological (European Type) No. 1, Neogen Corp.) in Petri dishes (90 mm x 15 mm triple-vented, Sterilin® Ltd.,) at 23 °C. Conidia were harvested from the surface of the agar after 14 days incubation by agitation with a Drigalski spatula on the sporulating colonies to remove the conidia, which were collected in 10 ml sterile 0.05 % (v/v) Triton X-100 (Thermo Scientific). The resulting suspension was centrifuged for five minutes at 3000 g/rf, the supernatant removed, and the pellet rinsed twice with sterile 0.05 % (v/v) Triton X-100 to remove all fragments of mycelia. The concentration of stock suspension was determined by counting conidia from 1000x serially diluted stock suspension in a Fuchs Rosenthal haemocytometer (×400 magnification) and diluted to required concentrations for experiments.

To assess the viability of conidial suspensions, a diluted suspension was prepared from each stock suspension, to give a concentration of $2x10^6$ conidia per ml, using sterile 0.05 % (v/v) Triton X-100. To verify conidial germination, 100 μL of 100x diluted stock suspension was spread with a Drigalski spatula on an agar plate of SDAY/4, sealed with Parafilm™ and incubated for 24 h at 23 °C. Four microscope coverslips (22 mm × 22 mm) were then placed on the culture surface and 100 conidia were counted under each coverslip. Conidia were considered to have germinated when the germ tube was at least as long as the width of the conidium. Conidial germination after 24 h was verified as > 98 % for all prepared inoculant suspensions before being used in assays.

2.3. Optimal and suboptimal temperatures for the host and pathogens

A range of four temperatures (18 °C, 23 °C, 28 °C, and 33 °C) were selected to construct growth curves for both the insects and fungi. These temperatures were chosen to examine both optimal and suboptimal temperature conditions that are predicted to lead to increased and decreased host and pathogen growth. The fungi have been routinely cultivated and maintained at 23 °C in the laboratory, a temperature to which we consider they are acclimatized. It has also been demonstrated that the optimal temperature for growth of *M. brunneum* is 24 – 25 °C, and it does not grow above 35 °C (Keyser et al., 2014; Kryukov et al., 2017). Conversely, the insects have been maintained at 28 °C, a temperature known to maximize growth in *T. molitor* (Eberle et al., 2022). Therefore, the temperature of 33 °C is predicted to be suboptimally high for both the host and pathogen, while 18 °C represents conditions that are suboptimally low for both host and pathogen.

2.4. Insect & fungal growth curves

To determine whether pathogens and host exhibit overlapping or distinct thermal performance curves and optima, we produced individual thermal profiles using growth rates across the four thermal treatments for each of the three organisms. To determine growth rates of *T. molitor* at these temperatures, 20 insects weighing between 250–850 mg were placed individually in vented square 25-compartment Sterilin® Petri dishes (100mmx100mm, Thermo Scientific, Basingstoke, UK) and provided wheat bran alone ad libitum and 0.2 g of 1 % (w/v) water agar twice weekly. Insects were incubated at 18 ± 1 °C, 23 ± 1 °C, 28 ± 1 °C, and 33 ± 1 °C, which were all maintained at a consistent relative humidity of 70 ± 5 %. Temperature and relative humidity were recorded every 15 min using EasyLog™ EL-SIE-2 dataloggers (Lascar Electronics Ltd., Salisbury, UK). Every five days over a 15-day period, the insects were weighed on an OHAUS Pioneer PA114CM Precision Balance (110 g / 1 mg, Ohaus Corp.). The experiment was replicated three times resulting in a sample size of 60 for each temperature treatment. To reveal how fungal infection moderated the host's response to the different temperature treatments, the growth rates of sublethally-infected *T. molitor* (see section 2.5) were measured following the same protocol.

Growth rates for each insect were derived from the slopes of linear models constructed for each insect as larval mass ~ time using base R version 4.2.3 (R Core Team, 2023). Pairwise comparisons of growth rates among temperature treatments were calculated based on the mixed-effects model slope ~ temp + (1|replicate) for each species, using the "lme4" package for constructing the models and the "multcomp" package for pairwise comparisons to apply Tukey's method for comparing a family of 4 estimates in R (Hothorn et al., 2008; R Core Team, 2023). For sublethally-infected insects, pairwise comparisons of growth rates were calculated in the same manner at each temperature using the model slope ~ species * dose + (1|replicate). Insects were included in the sublethal analysis if they survived to pupation.

To determine growth rates of fungal isolates at different temperatures independent of the host, fungal growth was measured using microspectrophotometry in 96 well plates using the technique described in Slowik et al. (2023). Microcultures were prepared in 42 wells of a clear flat-bottom vented microplate (Starlab International GmbH, Neuer Höltigbaum 38, Hamburg, Germany) by inoculating 100 μL of 2×10^6 conidial suspension into 100 μL quarter-strength Sabouraud dextrose + yeast liquid media (SDAY/4: 2.5 g/L 1:1 animal:bacterial peptone, 10 g/L dextrose, 2.5 g/L yeast extract). Microplates were incubated at 18 ± 1 °C, 23 ± 1 °C, 28 ± 1 °C, and 33 ± 1 °C, all at 70 ± 5 % relative humidity (RH). Temperature and RH were recorded every 15 min using EasyLog™ EL-SIE-2 dataloggers. The OD (optical density) of each well was measured at 405 nm in a SpectraMax 340PC microplate reader (Molecular Devices UK Ltd., Wokingham, UK) with SoftMax® Pro Version 3.0 software every 12 h from 24 – 84 h after inoculation during the linear growth phase of the fungus (Slowik et al., 2023). For each temperature, three replicates of 42 wells per treatment were prepared and measured, yielding a sample size of 126 per fungus isolate and temperature. To mitigate potential edge effects arising from temperature and evaporation, the 36 wells around the edge of the plate were excluded from analysis and loaded with 200 μL of blank media (Mansoury et al., 2021). The OD values of all microplates were also measured after conidia settled (15 min post-inoculation) to establish the baseline reading OD of each culture. This baseline reading was subtracted from subsequent measurements to determine change in OD for the

construction of growth curves.

To construct growth curves for the two fungal isolates at each temperature, linear models were fitted for each fungal culture as OD ~ time. Pairwise comparisons of growth rates among temperature treatments were calculated based on the mixed-effects model slope ~ temp + (1| replicate) for each species, using the "lme4" package for constructing the models and the "multcomp" package for pairwise comparisons to apply Tukey's method for comparing a family of 4 estimates in R (Hothorn et al., 2008; R Core Team, 2023).

2.5. Virulence thermal performance curves of the insect-fungus interaction

To investigate the effect of individual host-pathogen thermal performance curves on virulence and disease outcomes, we performed virulence assays across the four thermal treatments in *T. molitor* using two doses each for *M. brunneum* and *M. flavoviride*. To determine the effective doses for the host survival assay, initial virulence assays were carried out with the two fungal isolates to determine their lethal concentration LC50 and LC25 at 28 °C, which represent conidial concentrations expected to induce 50 % and 25 % mortality respectively after 20 days (Supplementary materials and methods). For *M. flavoviride*, the effective lethal concentrations LC50 and LC25 were at $8.7x10^5$ and $2.4x10^5$ spores/mL when 2 μL of inoculum was applied to the metathorax, resulting in the delivery of approximately 1,740 and 480 spores, respectively. For *M. brunneum*, the values were $4.55x10^5$ spores/mL (LC50; 910 spores) and $1.2x10^5$ spores/mL (LC25; 240 spores). The lethal concentrations for the *M. flavoviride* isolate KVL 114–12 were almost twice that of *M. brunneum* (KVL 12_30), indicating the infective capacity of *M. brunneum* was greater than *M. flavoviride* by a factor of 1.9, which follows the findings of Keyser et al. (2016).

Conidial suspensions for both isolates were then prepared using previously outlined methods for preparing inoculum. The experiments were carried out in vented 25-compartment Sterilin® Petri dishes (100x100mm, Thermo Scientific, Basingstoke, UK), each housing 20 separated insects weighing between 250 – 850 mg. A 2 μL droplet of the conidial suspension was applied to the metathorax of individual insects. Control insects received a 2 μL droplet of 0.05 % v/v Triton X-100. Post-inoculation, dishes were lined with uniformly moistened round 10 mm filter papers (1 mL DI water) and sealed with Parafilm™. These setups were incubated at 28 °C and 70 ± 5 % relative humidity for 24 h. Following this, filter papers and Parafilm™ were removed, and the insects were provided wheat bran ad libitum and 0.2 g of 1 % (w/v) water agar twice weekly and incubated at the four constant treatment temperatures, 18 ± 1 °C, 23 ± 1 °C, 28 ± 1 °C, and 33 ± 1 °C, all at 70 ± 5 % relative humidity in complete darkness. Temperature and relative humidity were recorded every 15 min using EasyLog™ EL-SIE-2 data-loggers. Larval mortality was recorded every two days for 20 days post-inoculation, and insects were weighed every five days on an OHAUS Pioneer PA114CM Precision Balance (110 g / 1 mg, Ohaus Corp.) to obtain growth rates during sublethal infections. Mortality due to fungal infection was confirmed through morphological observation of *Metarhizium* sporulation on cadavers. For this, cadavers were surface sterilized through a 20-second immersion in 5 % v/v bleach solution and two subsequent DI (deionized) water rinses. Cadavers were then enclosed in tubes with moistened cotton wool and incubated at 23 °C. No fungal infection was observed in any of the control treatments and containers for insect cadaver incubation were sealed to prevent cross-contamination. For the sublethal growth analysis, only weights of larvae surviving the experiment (20 days) were included. This experimental regime was replicated three times, yielding n = 60 per temperature and dose treatment.

To compare host survival outcomes for different temperatures, fungi and doses, survival models were constructed for each isolate at both doses using Cox proportional hazards regression in the R packages "coxme" and "survival" (Cox, 1972; Therneau, 2022; Therneau, 2023; R Core Team, 2023). Models were defined as (day, status) ~ treatment + temperature + (1 | replicate) for all four models. Pairwise comparisons for differences in survival probabilities among temperature treatments were calculated using Tukey's HSD test on the ANOVA of each model with the package "multcomp" in R (Hothorn et al., 2008; R Core Team, 2023).

3. Results

3.1. Thermal profiles of insect host and fungal pathogens

To assess the thermal sensitivity of *T. molitor* in terms of performance as growth, we measured growth rate and mortality at four different temperatures: 18 °C, 23 °C, 28 °C, and 33 °C (Fig. 1). The overall linear model indicated significant differences in growth rates across temperatures ($p < 0.0001$) (Supp. Table 1). Pairwise comparisons using Tukey's post-hoc tests revealed that growth rates at 18 °C and 23 °C were different from all others, although there was no significant difference in insect growth rate between the two highest temperatures, 28 °C and 33 °C (Fig. 1, Supp. Figure 1, Supp. Table 2). The significant differences in growth rates at varying temperatures suggest that *T. molitor* has a defined thermal profile for optimal growth. Growth rates at 28 °C that are significantly higher than at 18 °C and 23 °C likely reflect the physiological constraints of homeostatic processes in *T. molitor*, which are more efficient within a moderate thermal range. The absence of significant differences in growth rates between 28 °C and 33 °C may indicate an upper thermal threshold for *T. molitor* growth is approached at these temperatures. Beyond this threshold, the biological processes supporting growth may become heat-stressed or reach a plateau where further increases in temperature do not translate to enhanced metabolic efficiency or growth.

We assessed the thermal sensitivity of two *Metarhizium* species, *M. brunneum* and *M. flavoviride*, by measuring their growth rates across a range of temperatures that aligned with those of their insect host, *T. molitor*. Both species displayed significantly different growth rates across the temperature range, with the highest growth rates observed at 28 °C for both species ($p < 0.001$; Fig. 2A, B; Supp. Tables 3–7). However, the response to temperature differed between the two species. In *M. brunneum*, there was a threshold effect, with growth rates increasing from 18 °C to 28 °C, and then significantly decreasing at 33 °C (Fig. 2B). On the other hand, *M. flavoviride* showed a different pattern, with the second-highest growth rate at 18 °C (Fig. 2A). While both pathogens exhibited optimal growth at 28 °C, their responses to suboptimal

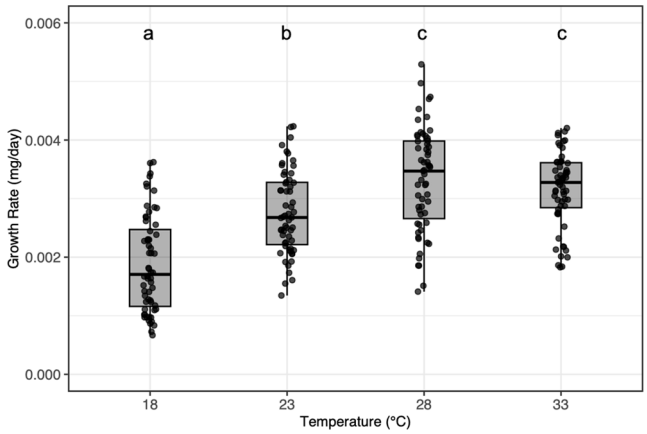

Fig. 1. Temperature-dependent growth of *T. molitor*. Growth rates of *T. molitor* at different temperatures, where each box plot illustrates the distribution of average growth rates for insects within a specific temperature group. Points indicate growth rates of individual insects. Letters represent significant differences in growth rates from pairwise comparisons between temperatures at the $p < 0.05$ level.

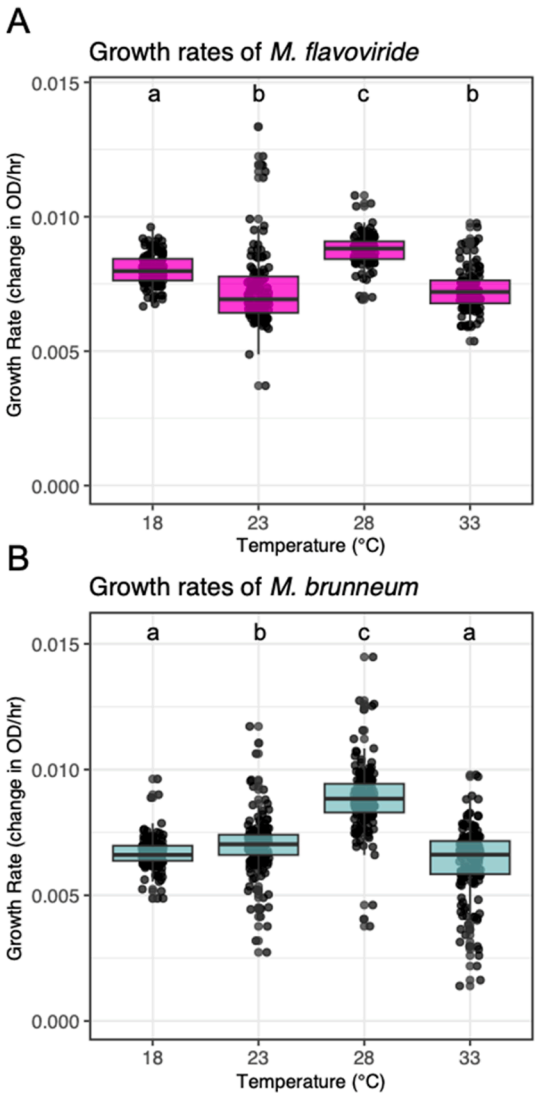

Fig. 2. Temperature-dependent growth rates of *Metarhizium* spp. Growth rates of *Metarhizium flavoviride* (**A**) and *Metarhizium brunneum* (**B**) across a range of four temperatures. Each box plot depicts the distribution of average growth rates for cultures within a specific temperature group. Points indicate growth rates of individual cultures in microplate wells. Letters represent significant differences in growth rates from pairwise comparisons between temperatures at the $p < 0.05$ level.

temperatures varied. *Metarhizium brunneum* showed a decrease in growth rate as the temperature deviated from the optimum, whereas *M. flavoviride* exhibited a narrower optimal niche, with consistent growth at lower temperatures and a sudden increase to optimal growth at 28 °C.

3.2. Virulence thermal profiles

In this section, we examine the virulence thermal profiles of both *Metarhizium* species, evaluating the effects of different temperatures on their ability to induce mortality in *T. molitor*. To construct these profiles, we first performed a survival analysis to determine survival probabilities for both pathogens at each temperature using two doses, LC25 and LC50. We found a significant effect of pathogen treatment for *M. brunneum* at LC25 ($\chi^2 = 57.6$, df = 3, $p < 0.0002$) and LC50 ($\chi^2 = 105.7$, df = 3, $p < 0.0001$) as well as *M. flavoviride* LC25 ($\chi^2 = 23.8$, df = 3, $p < 0.001$) and LC50 ($\chi^2 = 62.2$, df = 3, $p < 0.0001$) (Supp. Tables 8–15). This implies that both fungi can infect and kill the host at both doses. The overall model results did not indicate a significant effect of temperature on survival when looking at all temperature levels simultaneously, however the a priori pairwise comparisons revealed significant effects between specific temperatures (Fig. 3, Supp. Tables 8–15). The pairwise comparisons for survival among temperatures revealed no significant difference at the lower dose (LC25) for *M. flavoviride* (Fig. 3A). Planned pairwise comparisons revealed significant effects between temperatures in the various treatments (Fig. 3). For *M. brunneum* at LC25, the survival rate at 33 °C was significantly lower than at 23 °C ($p = 0.04$), and lower at 28 °C, but not significant ($p = 0.057$) (Fig. 3C, Supp. Tables 8). At higher doses (LC50), the only significant difference in survival for *M. brunneum* was between 33 °C and 23 °C ($p = 0.03$) (Fig. 3D). For *M. flavoviride* at LC50, we observed a greater differentiation in survival between temperatures; 28 °C caused significantly higher mortality than both 33 °C and 23 °C, although it did not significantly differ from 18 °C (Fig. 3B). The virulence rates at 23 °C and 33 °C were equally suboptimal for *M. flavoviride* at LC50 (Fig. 3B). Taken together, the significant effects observed from pathogen treatment on survival rates, as indicated by the GLM, reflect the biological impact of the pathogens' virulence at different doses and temperatures on *T. molitor*. For *M. brunneum*, the significant temperature effects at both LC25 and LC50 suggests a high virulence potential across the temperature range tested, which may correlate with its broader host range and environmental versatility. In contrast, the lack of a significant temperature effect for *M. flavoviride* at LC25 may suggest a more stable virulence across the tested temperatures. However, the increased mortality at 28 °C, could imply that *M. flavoviride's* virulence is optimized at this temperature, which may align with its individual thermal niche, allowing for more effective infection and proliferation.

Adding to the thermal profiles of *T. molitor* and the two *Metarhizium* species, we investigated the growth rates of *T. molitor* larvae that survived sublethal infections by either *M. brunneum* or *M. flavoviride* and subsequently reached pupation (Fig. 4, Supp. Tables 16–23). The growth rates of these sublethally infected insects provided insights into the effects of overcoming infection and its interaction with environmental temperature. We observed no significant differences in growth rates among the pathogen treatments at 18 °C (Fig. 4A) in the pairwise comparisons, indicating negligible effects of the suboptimal temperature or pathogen treatment on larval growth at this lower temperature. Conversely, at the acclimated temperature of 23 °C, the control group had significantly higher growth rates compared to the LC50 treatment groups of both fungi (F50 – Control $p = 0.025 \pm 0.0002$, df = 210; B50 – Control $p = 0.032 \pm 0.0002$, df = 210). Growth rates in the LC25 groups were reduced compared to the control, but were not statistically different from either the control or the LC50 treatments (Fig. 4B). This pattern indicates that growth rate was influenced by the pathogen dose used rather than the pathogen species, although exposure to *M. flavoviride* consistently resulted in lower mean growth rates than exposure to *M. brunneum* at 23 °C, 28 °C, and 33 °C (Fig. 4). At higher temperatures of 28 °C (acclimated) and 33 °C (suboptimal) (Fig. 4C, D), the growth rates in control groups did not significantly differ from the LC25 groups. However, the *M. flavoviride* LC50 group presented a distinct response, showing significantly lower growth rates from the control group, but not differing from other treatment groups (at 28 °C, F50 – Control $p = 0.04 \pm 0.0002$, df = 203; at 33 °C F50 – Control $p = 0.01 \pm 0.0002$, df = 240).

After determining growth rates for each individual organism and assessing survival rates of *T. molitor* infected with each pathogen across temperatures, we integrated these findings to construct thermal profiles for the three species individually and the virulence thermal profiles of the two fungal species. Temperature-dependent virulence was shown by comparing the growth of *T. molitor* (Fig. 5A) and the growth of the two fungi, *M. brunneum* and *M. flavoviride* (Fig. 5B) with the virulence thermal profiles of fungi (Fig. 5C). Thermal growth performance was similar for the pathogens *M. brunneum* and *M. flavoviride* and the host *T. molitor* (Fig. 5A, B), as they share a thermal optimum at 28 °C albeit

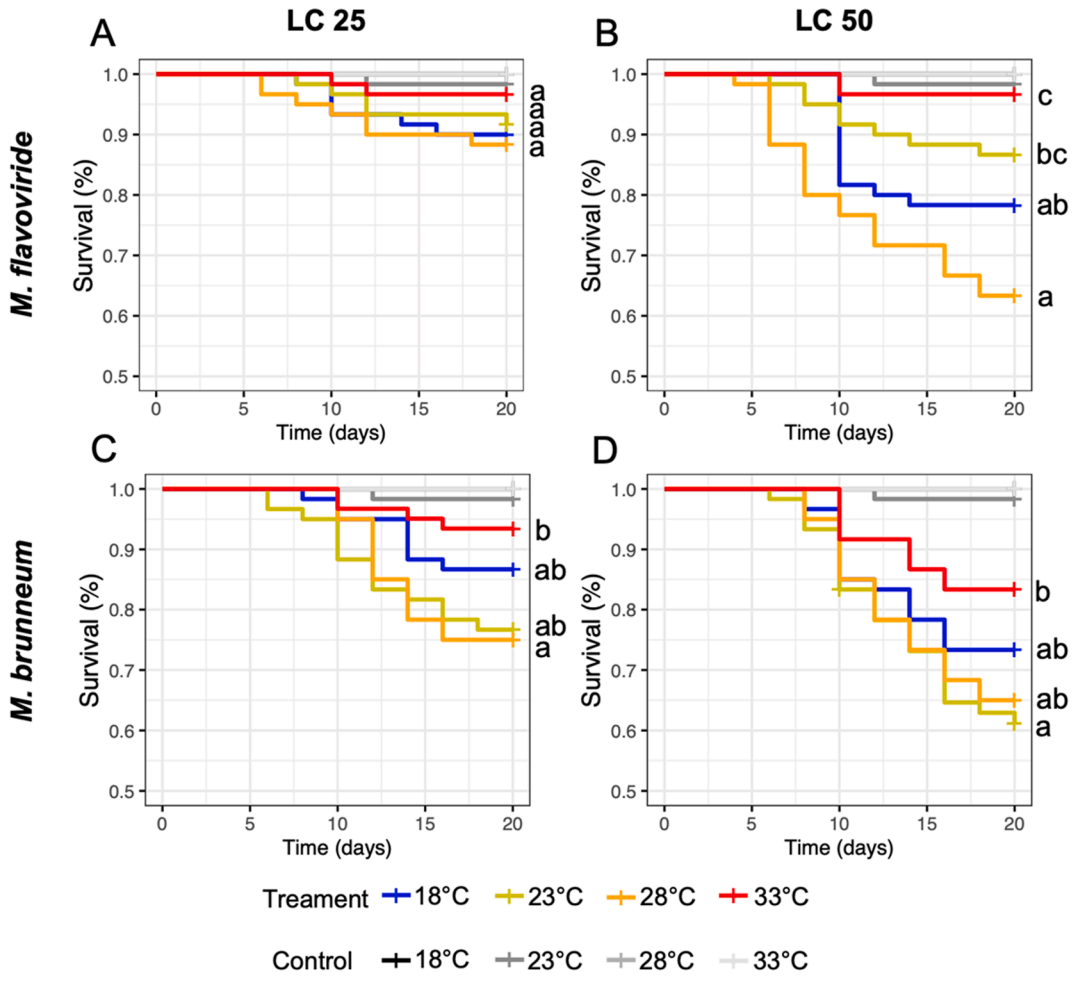

Fig. 3. Temperature-dependent survival of *T. molitor* infected with *Metarhizium* spp. Survival probabilities of *Tenebrio molitor* infected with two species of *Metarhizium* fungi at two doses, LC25 and LC50, across a range of four temperatures from 18 °C to 33 °C. Letters represent significant differences in survival from pairwise comparisons between temperatures at the $p < 0.05$ level.

with a steeper growth peak for *M. brunneum* around this temperature. The fungus *M. flavoviride* in addition show increased growth at the lowest temperature 18 °C, which is expected based on the known biology of this species (Driver et al., 2000). Considering both the thermal and virulence performance profiles, there was a common thermal optimum at 28 °C for virulence for both pathogens and growth in all three species (Fig. 5A, B, C). In *M. flavoviride*, the thermal profile closely aligned with the virulence profile, indicating that temperature has a similar effect on both growth and virulence (Fig. 5B, C). Conversely, the reaction norm for *M. brunneum* diverged from the thermal profile for growth at 23 °C, where virulence was optimized, and showed that the interaction of the host and the pathogen at this sub-optimal temperature for both, led to increased virulence (Fig. 5A, B, and C). These results collectively suggest that temperature has an important effect on the outcomes of interactions between these organisms, although the effects are not uniform across the different species.

4. Discussion

This study explored the thermal performance curves of the entomopathogenic fungi *M. brunneum* and *M. flavoviride*, and a model insect host *Tenebrio molitor*, at their acclimated temperatures (23 °C and 28 °C, respectively), and during both hot (33 °C) and cold (18 °C) sub-optimal temperatures. We found that both the uninfected and sublethally infected insect hosts and fungal pathogens grew optimally at 28 °C and that virulence of *M. flavoviride* was correlated with its temperature profile, whereas virulence for *M. brunneum* was also increased at 23 °C, but its growth was not. These findings provide insights into the alignment of thermal performance optima, revealing that the effect of temperature on entomopathogenic fungal interactions is not uniform across pathogen species with different ecological niches and host ranges (Mongkolsamrit et al., 2020), and highlights that past evolutionary history of host-pathogen interactions may influence the outcome of infections experiencing a thermal mismatch of host and pathogen.

The shared thermal optima at 28 °C for individual growth across all three species suggested temperature alignment and minimal thermal mismatch. However, the complexity in virulence patterns, e.g., *M. brunneum*'s broader virulence thermal niche compared to its thermal profile for growth, revealed interactions influenced by factors beyond thermal optima for the single trait of growth in the host and pathogens. While there is an obvious impact of temperature on disease outcomes, the different responses observed in the two pathogens suggest that the TMH might have variable applicability based on the specific evolutionary and ecological contexts of the interacting organisms. Larval growth when sub-lethally infected highlighted the dynamic interactions between the pathogen dose and the influence of temperature on the host. Except at the coldest temperature of 18 °C where there was no effect on growth, sublethal infections resulted in reduced growth of the host. This effect was highest for LC50 treatments and therefore was dose-dependent, but there was also a consistent but non-significant trend of more reduced growth of larvae with a sublethal infection with *M. flavoviride* compared to *M. brunneum*. This implied that sublethal

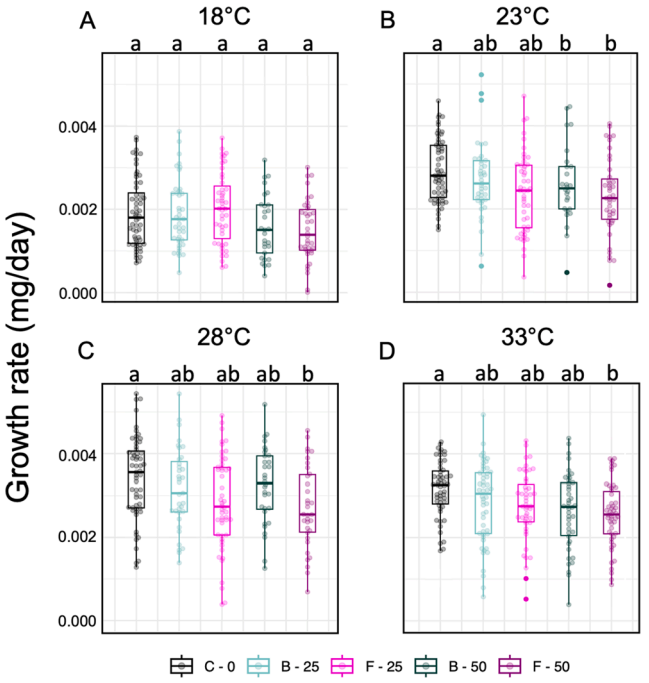

Fig. 4. Growth rates over 15 days of *Tenebrio molitor* larvae at varying temperatures post sublethal infection with *Metarhizium* spp. Panels (A-D) correspond to different temperature settings: 18 °C (A), 23 °C (B), 28 °C (C), and 33 °C (D). Within each panel, box plots illustrate the growth rates (mg/day) of larvae across different treatments: control (C-0), *M. brunneum* at LC25 (B-25), *M. flavoviride* at LC25 (F-25), *M. brunneum* at LC50 (B-50), *and M. flavoviride* at LC50 (F-50). The central line of each box plot represents the median growth rate, the box boundaries indicate the interquartile range (IQR), and the whiskers extend to the furthest points within 1.5 times the IQR from the box. Points represent individual larval growth rates. Different letters above the box plots indicate statistically significant differences in growth rates between treatment groups within the same temperature, based on pairwise comparisons.

fungal infections reduced growth at warmer temperatures, but this effect was possibly masked by the severity of the cold temperature treatment at 18 °C. Considering the survival outcomes, the data show that temperature affects both the overall outcomes of host-pathogen interactions (mortality) and the subsequent host recovery and growth. These findings reveal that sublethal infections impose fitness costs on the host, evident through diminished growth rates, which could negatively affect the insects' reproductive success or other physiological processes and possibly pathogen transmission, should the host eventually succumb to the infection.

Previous studies have examined the effect of temperature in EPF in the context of biocontrol, on traits such as germination and virulence (Brunner-Mendoza et al., 2019; Couceiro et al., 2021; Seib et al., 2023; Velavan et al., 2022). Here we capture the thermal sensitivity of both the host and pathogen individually as well as during the interaction of the host and pathogen at suboptimal temperatures. According to the thermal mismatch hypothesis (TMH), we anticipated peak virulence at colder temperatures where the warm-adapted host *T. molitor* performs suboptimally, but where the pathogenic fungi would maintain high virulence (Cohen et al., 2020; Rohr & Cohen, 2020). In the thermal virulence profile of *M. brunneum*, this was indeed what we observed with a broader thermal niche for virulence, which includes the thermal optima of the host (Fig. 5A,C). In comparison, in the more cold-adapted *M. flavoviride* there is a narrower thermal niche for virulence, which aligns with that of the host's individual growth optima at 28 °C (Fig. 5C). Both host and pathogens have been acclimated to specific temperatures through continued maintenance in laboratory conditions over many generations. The adaptation of the different pathogens according to

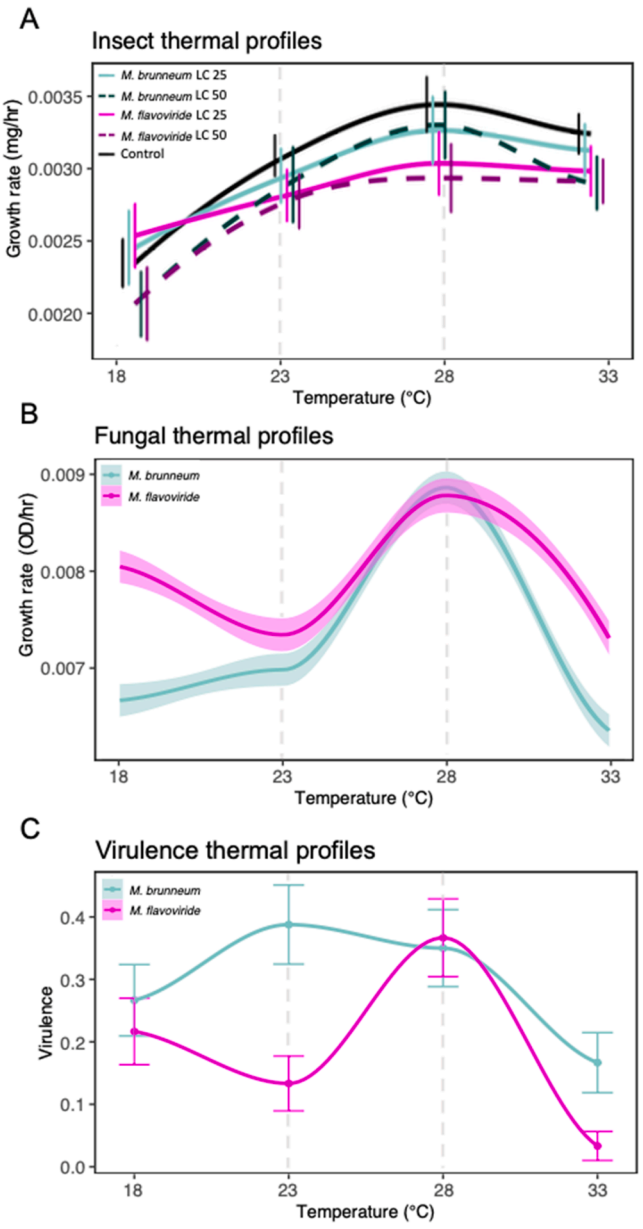

Fig. 5. Thermal responses in host and pathogen growth and virulence dynamics. **A.** Thermal performance curves of *Tenebrio molitor,* differentiated by species and dose treatments. Error bars indicate 95 % CI modeled from data in Figs. 1 and 4. **B.** Thermal performance curves of *M. brunneum* and *M. flavoviride* based on growth rates in Fig. 2. Shaded bands indicate 95 % CI. **C.** Temperature-dependent virulence performance for *M. brunneum* and *M. flavoviride.* Virulence is defined as the likelihood of *Tenebrio* mortality after 20 days of infection at LC50 for each pathogen. Points indicate predicted probability of mortality for *Tenebrio* at day 20, bars are SE for these estimates.

their ecological evolutionary trajectories could have led to asynchronous development in thermal niches, where both organisms might have developed unique mechanisms to cope with thermal stress, leading to an unconventional pattern of virulence. For instance, while virulence of *M. brunneum* aligns with the TMH, the thermal performance patterns of *M. flavoviride* diverge from TMH predictions. Such divergence is reflective of a broader ecological phenomenon where species respond asymmetrically to climatic factors, a concept supported by findings in both plant-herbivore and host-pathogen systems (Paudel et al., 2020; Porras et al., 2023). Paudel et al. (2020) described how temperature differentially affected insect herbivore and host plant responses, leading to an asymmetry in responses that was further complexed by the

interaction between herbivore-associated elicitors of plant defense. Similarly, Porras et al. (2023) demonstrated that extreme heat events impacted the performance of both insect hosts and their pathogens, reducing the heat tolerance of hosts and the growth rate of pathogens. These studies underscore the complex and often species-specific impacts of temperature on biological interactions and suggest that the responses of *M. flavoviride* and *M. brunneum* to temperature stress may be part of a wider spectrum of thermal ecological strategies. Such asymmetrical responses to thermal stress noted in our fungal species might reflect evolutionary pressures that have shaped unique virulence strategies, which could have significant implications for the dynamics of host-pathogen interactions in the context of a changing climate.

The shared thermal optima for the host and *M. flavoviride* in terms of growth and virulence could be a result of shared adaptive traits or environmental constraints. EPF and their insect hosts might have similar environmental pressures affecting them, given that they share the same soil environment (St Leger & Wang, 2020). Thus, soil insects and fungi may adapt to these pressures in ways that align their thermal optima. Specifically, soil-dwelling *Metarhizium* species appear adapted to their environmental habitat in the soil and plant root associations rather than insect hosts (Hu & Bidochka, 2021; Keyser et al., 2014; St Leger & Wang, 2020), with an ecological strategy of exploiting short lived, transitory insect hosts, and forming a beneficial mutualistic relationship with plants which provide a stable environment (St Leger & Wang, 2020). The interplay of the evolutionary forces in the shared soil and plant-root environmental niche could yield shared adaptive thermal traits, leading to a convergence in how both host and pathogen respond to thermal stress, forcing them into a shared optimum.

It is important to consider that the concept of thermal adaptation is tied intrinsically to the specific traits being evaluated. For example, there could be indirect effects and trade-offs that were not captured in this study that would expand our understanding of the system. The interplay of various adaptive traits, each responding to temperature differently, complicates our understanding of thermal adaptation. Considering this, there are indications that *T. molitor* may not be strictly warm-adapted. Whilst growth is maximized at elevated temperatures, it does not encapsulate the entirety of the organism's adaptive traits. In natural environments, *T. molitor* can overwinter and endure extended periods at low temperatures (Graham et al., 2000). Furthermore, choice experiments with *T. molitor* show a preference for temperatures well below 28 °C, indicating potential trade-offs and underlying physiological or ecological advantages linked to cooler environments (Catalán et al., 2012). The observed patterns of *Metarhizium* spp. virulence and thermal responses are likely idiosyncratic to the specific isolates under study, given that these fungi exhibit extensive intraspecific variation among isolates for performance in virulence and temperature (Driver et al., 2000; Reingold et al., 2021; Couceiro et al., 2021; Tong & Feng, 2020). A broader exploration of traits important to virulence such as conidia production and host immune responses (e.g. Maistrou et al., 2020; Lu and St. Leger, 2016), for a diverse array of isolates and an expanded temperature range, would be instrumental in drawing more generalized conclusions. However, this study provides a framework for detecting different virulence and growth patterns outside of the thermal range investigated.

It is also important to note that during the initial 24 h of the infection process, we maintained the experimental conditions at the insect's optimal temperature of 28 °C with high humidity to ensure infection, as this is crucial for initial pathogen establishment. During this early phase, the pathogens and host were not subjected to the varied experimental temperatures. This methodological nuance, while necessary to ensure successful infection, may have influenced the early interaction dynamics between the pathogens and the host, and as such is a critical consideration in interpreting the thermal performance curves we observed.

The differences in how the individual thermal profile for each pathogen correlated with their respective thermal virulence profile was noteworthy for *M. brunneum*. This species is recognized for its ecological and pathogenic versatility that allows it to infect multiple insect orders and grow symbiotically with many plant hosts (St Leger & Wang, 2020; Moonjely & Bidochka, 2019; Hu & Bidochka, 2021). Conversely, *M. flavoviride* which is considered to be a less versatile pathogen primarily isolated from *Coleoptera* hosts and from soil (Keyser et al., 2016; Meyling et al., 2011), had a narrower optima, which corresponded with its individual thermal profile. The alignment of growth and virulence thermal optima suggests that the thermal constraints and adaptations intrinsic to *M. flavoviride* may directly impact its virulence. The distinct responses exhibited by *M. brunneum* and *M. flavoviride* to temperature variations highlight the role of species-specific adaptations in determining infection outcomes.

In this study *M. flavoviride* exhibited two peaks in virulence, one critically at a lower temperature, and it also grows relatively well at lower temperatures (Driver et al., 2000). This is in concordance with *M. flavoviride* having a distinct ecology, which may be more locally adapted to performing well at lower temperatures as a weakly infective root symbiont, opportunistically infecting specific insects in the rhizosphere. We found *M. brunneum* to be twice as virulent in terms of number of spores needed to induce mortality, with a broader virulence thermal performance curve. This indicates *M. brunneum* is capable of infecting and killing insects over a broader range of temperatures beyond the individual thermal niche for growth for this species. The fungus *M. brunneum* is considered to be a pathogen with a broad host range of insects, which could be due to its evolutionary history with fungi in the PARB-clade having acquired virulence genes through horizontal gene transfer (Zhang et al., 2019).

Our findings highlight the complex nature of thermal adaptation and its implications on the interactions between entomopathogenic fungi and their insect hosts. The variance in virulence, despite shared thermal growth optima, underscores the need to consider the specific ecological and evolutionary contexts of each species when incorporating broad theories, such as the Thermal Mismatch Hypothesis.

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CRediT authorship contribution statement

Anna R. Slowik: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Helen Hesketh:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Steven M. Sait:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Henrik H. De Fine Licht:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2024.108106.

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