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Fungal infection dynamics in response to temperature in the lepidopteran insect *Galleria mellonella*

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Abstract

This study examines how the dynamics of fungus – insect interactions can be modulated by temperature. The wax moth, *Galleria mellonella*, is a well-studied and important model insect whose larvae in the wild develop optimally at around 34°C in beehives. However, surprisingly little research on wax moths has been conducted at relevant temperatures. In this study, the entomopathogenic fungus *Metarhizium robertsii* inflicted rapid and substantial mortality on wax moth larvae maintained at a constant temperature of 24°C, but at 34°C a ten-fold higher dose was required to achieve an equivalent mortality. The cooler temperature favored fungal pathogenicity, with condial adhesion to the cuticle, germination and hemocoel invasion all significantly enhanced at 24°C, compared with 34°C. The wax moth larvae immune responses altered with the temperature, and with the infective dose of the fungus. Enzyme-based immune defenses (lysozyme and phenoloxidase) exhibited enhanced activity at the warmer temperature. A dramatic upregulation in the basal expression of galiomicin and gallerimycin was triggered by cooling, and this was augmented in the presence of the fungus. Profiling of the predominant insect epicuticular fatty acids revealed a 4–7-fold increase in palmetic, oleic and linoleic acids in larvae maintained at 24°C compared with those at 34°C, but these failed to exert fungistatic effects on topically applied fungus. This study demonstrates the importance of choosing environmental conditions relevant to the habitat of the insect host when determining the dynamics and outcome of insect / fungus interactions, and has particular significance for the application of entomopathogens as biocontrol agents.

Key words biocontrol; entomopathogenic fungus; immunity; *Metarhizium*; temperature

Introduction

There is much interest in the use of entomopathogenic fungi (EPF) for the control of arthropod pests, with over 66% of the commercial products being based on strains of *Metarhizium anisopliae* s.l. and *Beauveria bassiana* s.l. (Faria & Wraight, 2007). EPF efficacy is influenced by temperature and the host response to the pathogen. High temperatures (> 30^{o}C) can impede fungal growth and may explain why some insects exhibit behavioural thermoregulation. This behaviour usually entails basking in the sun to elevate body temperatures to levels that are detrimental to the pathogen (De Roode & Lefevre, 2012). Such fever responses have been observed in several insect species including locusts, grasshoppers, flies, and bees (Blanford & Thomas, 1998; Starks *et al*., 2000; Klasbeek, 2001; Ouedraogo *et al*., 2004). These changes in thermal regime usually favor the immune response and promote host survival, but not all insects exhibit behavioral thermoregulation, and where it is reported it is often at a cost to the host (Blandford *et al*., 2009; Anderson *et al*., 2013a,b). Some insects seek lower temperature environments that presumably slow down fungal development, giving the insect more time to mount an effective defense response to resist the pathogen (Hunt *et al*., 2016). Quite a few insects do not regulate temperature but their survival is vastly improved if kept at higher temperatures, as reported for the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) exposed to *Metarhizium brunneum* (Fisher & Hajek, 2014).

The Greater wax moth, *Galleria mellonella* (L.) is susceptible to EPF and non-EPF and is used as a bait to isolate EPF from the soil (Zimmermann, 1986; Wakil *et al*., 2013). In apiculture, the wax moth is considered to be a pest because the larvae feed on the honeycomb inside beehives. The temperature in the beehive brood nest ranges between 32 and 36^{o}C (Kronenberg & Heller, 1982; Becher & Moritz, 2009), and it is important to note that wax moth larvae maintained in laboratory

culture boxes can generate temperatures between 32 and 40°C even when the external (room) temperature is maintained at 28°C (Buchmann & Spangler, 1991; Schmolz & Schulz, 1995). This temperature range it is not optimal for fungal growth and may explain why the larvae escape infection. For most EPF, including thermophilic strains, growth slows down or ceases between 30°C and 40°C (Rangel *et al.*, 2010; Kryukov *et al.*, 2012). Although they can tolerate brief exposure to even higher temperatures (e.g. 50°C) for most strains 30°C is the upper temperature threshold for growth (Ekesi *et al*., 1999).

The role of the wax moth's cuticular, cellular and humoral defenses in resisting fungal infection has been studied at ambient temperature (Dubovskiy *et al*., 2013a,b). In contrast, little is known about the competency of these defenses at the higher temperatures favoring the moth's development. Brief exposure of waxmoth larvae to 38°C for 30 min before injection with *B. bassiana* blastospores or exposure of the naturally infected animals to abnormally high 43°C for 15 min extended their lifespan (Wojda *et al*., 2009). The improved survival was attributed to higher expression of antimicrobial peptides and higher antifungal and lysozyme activities in the hemolymph of heat shocked animals (Wojda *et al*., 2009). Although continuous exposure of wax moth larvae to 43°C is lethal, the insects will, upon recovery from brief heat shock (15 min), exhibit an enhanced antifungal response manifested in upregulation of Apolipophorin III (Vertyporokh *et al*., 2015). Exposure of larvae to 12°C (cold shock) before infection with an entomopathogenic bacterium also resulted in a stronger immune response than for insects permanently reared at 28°C (Wojda *et al*., 2015). As far as we are aware, no study has been conducted of the wax moth defense response to entomopathogenic fungi at the sustained elevated temperatures normally encountered in bee hives. However, there are several studies on infection of the larvae by human pathogens with most studies

being conducted at/below 37°C and with the microbes being injected into hemocoel (e.g. Melo *et al.*, 2013; Gago *et al.*, 2014; Tsai *et al.*, 2016) i.e. cuticular defense was not examined. The cellular immune response is mediated by hemocytes which encapsulate and nodulate invasive microorganisms (Dubovskiy *et al.*, 2016). Humoral defenses include phenoloxidase (PO), which synthesizes melanin which can inhibit fungal growth, and antimicrobial peptides including gallerimycin and galiomicin which are known to exhibit antifungal activity (Butt *et al*., 2016). Besides the cellular and humoral defenses, EPF have to overcome the inhibitory compounds in the epicuticular waxes (Butt *et al*., 2016). Most often, resistance has been attributed to fungistatic fatty acids, with caprylic and capric acid amongst the most commonly reported in arthropods (e.g., Koidsumi, 1957; Smith & Grula, 1982; Saito & Aoki, 1983; Urbanek *et al*., 2012). The nature and composition of the epicuticular waxes will alter at elevated temperatures (Gibbs, 2002) and any changes could influence conidial attachment and other stages of the infection process. This study investigated the infection of wax moth larvae by the EPF, *Metarhizium robertsi* (formerly *M. anisopliae*), and the host defense response at sustained optimal (34°C) and less optimal (24°C) temperatures.

Materials and methods

Maintenance of wax moth

Laboratory populations of the wax moth, *Galleria mellonella* reared at Swansea University (UK), were maintained on artificial medium (Dubovskiy *et al*., 2013a) in an insectary with an ambient temperature of 28^{o}C. In the breeding colony, the gregarious nature of the larvae results in large insect clusters, the interior temperature of which greatly exceeds that of the room. Validation of the

cluster temperature was conducted using temperature logging devices, as outlined in the ESM. Fifth instar larvae were used in all subsequent experiments.

Fungal culture

Metarhizium robertsii P-72 was maintained on Sabouraud's Dextrose Agar (SDA) at 27°C. This strain, isolated from a Colorado Potato Beetle in Latvia 1972 (Serebrov *et al*., 2007), will grow between 10°C and 37°C but growth is 2.3-fold greater at 25 ± 1°C versus 35 ± 1°C as determined by preliminary radial growth experiments (see Supplemental information). Conidia were harvested from 14 day old sporulating cultures, air-dried at RT for 1 week and stored at 4°C until required.

Inoculation of wax moth larvae

For bioassays, conidia were suspended in sterile 0.03% (v/v) aqueous Tween 80 and diluted to final concentrations of 10^7 and 10^8 conidia/ml. Insects were inoculated by dipping individuals in the spore suspension at room temperature for 10 s, with control larvae being immersed in 0.03% aqueous Tween 80 only. Control and infected insects were kept at 24°C or 34°C in 9 cm diameter ventilated Petri dishes (10 larvae/dish) lined with moistened filter paper, and incubated for 9 days (until the pupal stage). It is important to note that in the physiological experiments described herein, the external temperature (i.e. either 24°C or 34°C) was the same as the internal temperature inside Petri dishes. This is because there were only 10 individuals per Petri dish. Each cohort of larvae was provided 3 g of food. Dishes were opened daily for 4-5 min to record the mortality. There were 70 larvae per treatment and the whole experiment was repeated 2 times.

Adhesion, germination and colonization assay

Conidial adherence was studied using methods adapted from Ment *et al*. (2010a). Briefly, conidia were removed from the cuticle of inoculated larvae 6 h pi by washing with 99% dichloromethane. Ethanol (99% purity) was added 1 : 1 to the dichloromethane and the conidia pelleted by centrifugation at 3000 g for 30 min at 25°C. After evaporation of the solvent, the conidia were suspended in 0.05% Tween-80 and counted using a hemocytometer. At least 8 larvae were examined per treatment.

To observe conidial adhesion and germination on insect cuticle, larvae inoculated as described above were fixed at 6, 24 and 48 h pi in 2% aqueous formaldehyde for 24-48 h at 4^{o}C. The specimens were washed with buffer/water before immersing in an aqueous solution of 0.1% Calcofluor white for 25 s, and air-dried before removing the head and hemocoel contents. Calcofluor white binds to fungal cell wall sugars, thus helping to visualize germlings. The detached cuticle was observed using an Axioscope 40 fluorescent microscope and the percentage germination was determined by observing 100 conidia per larva. At least 7 larvae from each treatment at each time point were examined.

To determine if the fungus had penetrated the cuticle and was circulating in the hemocoel, the proleg was cut and 10 μL hemolymph were mixed with 40 μL sterile anticoagulant solution containing 0.4% phenylthiourea to prevent melanization and clotting. Each sample was then inoculated onto SDA supplemented with 0.4% lactic acid (to suppress bacterial growth) and the agar

plates incubated for 3 d at 25°C to encourage fungal growth. The number of insects with systemic infections of *M. robertisii* was determined from plates exhibiting fungal colonies. Blood samples were taken from 20 larvae per treatment.

Fatty acids *composition of* Galleria *epicuticular waxes*

The epicuticular waxes play an important role in protecting insects against microbial agents and dehydration. For this reason, the fatty acid composition of the wax layer was determined using whole larvae incubated 24 h under 24°C and 34°C. Briefly, 3 independent groups of 20 larvae were immersed in 20 ml 99% dichloromethane and incubated in a rotary shaker (130 r/m) at 22°C for 5 min. Samples were taken to dryness under a stream of nitrogen before examination by GC-MS. Fatty acid methyl esters (FAMEs) were analysed by an Agilent 6890GC interfaced directly to an Agilent 5975 mass spectrometer (split/splitless injection, 80 : 1 split ratio; 70eV, EI). Separation was achieved by a HP-INNOWAX capillary column (30m × 0.32 mm; film thickness 0.25 μm). The oven temperature program was held at 120°C for 9 min, ramped at 20°C/min to 230°C where it was held for 10 min. The carrier gas was helium. Identification was achieved using the NIST spectral library and with comparison to published spectra. Quantification was achieved using a calibration curve constructed from known quantities of C_{17} FAME run using the same conditions as the samples. Peak areas were used.

Fungal germination on authentic fatty acids

The influence on germination of the fatty acids identified in the above study was investigated using pure compounds purchased from Sigma, UK. The methodology, adapted from Ment *et al*. (2010b, 2013), entailed dilution of the fatty acids in 99% n-hexane to a concentration of 1, 0.1 and 0.01% (w/v) then application of 50 μL to cover-slips. After solvent evaporation, the coverslips were placed upside down on 2% agar that had been pre-inoculated with *M. robertsii* conidia (10^6 per mL). In the control samples coverslips were treated with pure n-hexane. The agar plates were incubated for 24 h at 26°C, and the resulting percentage of germinated conidia were counted under a light microscope. At least 200 conidia from each of 3 replicates were observed.

Phenoloxidase activity

Larvae were inoculated with *M. robertsii* conidia as described above, and at 6, 24 and 48 h pi, cell-free hemolymph plasma samples and homogenized integuments were prepared for spectrophotometric analysis of phenoloxidase (PO) enzymatic activity (Dubovskiy *et al*., 2013a). L-3,4-dihyroxyphenylalanine dissolved in sterile pyrogen-free water was used as a substrate and PO activity was expressed as a change in absorbance/min/mg protein at 28°C. Uninfected insects were used as the controls. Individual larvae were used for each plasma sample, but each integument sample was pooled from three insects. At least 30 plasma (30 individual insects) and 10 integument samples (i.e. total 30 insects) were examined per treatment and per time point.

Encapsulation response

The strength of the hemocytic-melanotic encapsulation response towards a foreign implant was assayed 6, 24 and 48 h after fungal inoculation. A knotted piece of white nylon monofilament, 2 mm long and 0.5 mm in diameter, was injected into the hemocoel of each larva via perforation of a ventral segment of the cuticle. Implants were dissected from the body cavity two hours later and then photographed from three angles. The degree of melanization was quantified using Image Pro software by measuring the coloration (gray value) of all areas of each implant, and then comparing these values with that of an intact implant (without melanization) (Dubovskiy *et al.*, 2013a). One nylon implant was assayed per larvae tested and at least 25 larvae analyzed per treatment and per time point.

Plasma lysozyme-like activity

Lysozyme-like antibacterial activity of hemolymph plasma was determined by a radial zone of inhibition assay as described by Wojda *et al*. (2009). Briefly, larvae were inoculated as above, and at 6, 24 and 48 hpi, a 10 μL aliquot of hemolymph from an individual larva was collected into 2 μL ice-cold PBS containing PTU (4 mg/mL). The suspension was centrifuged at 500 g for 5 min at 4°C. Five microlitres of the resulting cell-free plasma supernatant were placed into a 1 mm diameter well in 1.5% agarose plates containing physiological solution (NaCl 0.9%) and 4 mg/mL freeze-dried *Micrococcus lysodeikticus* and incubated at 28°C for 18 h. Plasma lysozyme-like activity was observed as a clear zone of digested *M. lysodeikticus* peptidoglycan, the radius of which was measured and quantified using a standard curve of egg white lysozyme (EWL) according to Mohrig and Messner

(1968) and expressed as an EWL equivalent (mg/mL). One larva was used per sample and 15 samples were assayed per treatment and per time point.

AMP genes expression

Gallerimycin and galiomicin are key antimicrobial peptides (AMPs) synthesised by *G. mellonella*, for which strong antifungal activity has been demonstrated (Schuhmann *et al.*, 2003; Lee *et al.*, 2004). The effect of temperature on the expression of these AMP genes was assessed in larvae over a 3-day period, both with and without fungal infection, to assess both basal (uninfected) and induced (fungus-infected) transcript levels. Gene expression was measured in dissected fat body samples by real-time quantitative RT-PCR using normalized cDNA samples with the Rotor-Gene 6000 (Corbett Research), with Rotor-Gene SYBR Green PCR mix (Qiagen), relative to reference gene *Elongation Factor 1-alpha* (EF1; AF423811). Full details are provided in the SI.

Statistical analyses

Data analyses were performed using GRAPHPAD PRISM v. 4.0 (GraphPad Software, USA), STATISTIC v. 8.0 (StatSoft Inc., USA) and SigmaStat v. 3.1 (StatSoft Inc., USA). Data were checked for normal (Gaussian) distribution using the Shapiro-Wilk W test, and if abnormally distributed a more conservative non-parametric analysis was applied. Percentages data for conidial germination were subjected to arcsine transformation of the square root. A LogRank test was used for the insect survival analysis. Differences between the quantities of cuticular fatty acids were analyzed using the Mann-Whitney U test. The extent of fungal adhesion and germination on the cuticle was compared

by two-way analysis of variance (ANOVA) followed by Bonferroni test. The χ^2 test was used to compare the ratio of larvae that had or had not been colonized by the fungus. One-way ANOVA was used to assess differences between treatments and controls in the lysozyme and encapsulation response assays (with the Bonferroni post test), and in the PO assays (with the Kruskall-Wallis ANOVA with Dunn's post test). For QRT-PCR expression data exhibiting a Gaussian distribution, Grubbs' extreme studentized deviate (ESD) test was used to exclude any extreme outliers. Individual gene expression comparisons were made with the *t*-test and non-parametric one-way ANOVA (Kruskall-Wallis with Dunn's post test).

Results

Insect mortality

At 34°C (optimal for the wax moth but non-optimal for the pathogen), larval mortality was low (< 16% after 9 days) at the low dose of the fungus (10^7 conidia/ml), and similar to all the un-infected controls (Fig. 1; $\chi^2 = 4.789$, df = 2, $P = 0.09$). Although at this temperature mortality increased at the higher dose (10^8 conidia/mL), the fungus was more virulent at 24°C (less optimal for the insect but optimal for the pathogen) at both doses (Fig. 1).

At 24°C, the mycosis developed most rapidly at the higher inoculum dose ($LT_{50} = 3 \pm 0.08$ days pi, compared with 4 ± 0.17 days pi for the lower dose; $\chi^2 = 58.1$, df = 1, $P < 0.001$). In both cases, cumulative mortality achieved 95%–100% after 9 days pi, which was significantly higher than the

uninfected controls (Fig. 1; $\chi^2 > 118.6$, df = 1, $P < 0.0001$). At 34°C at the higher dose, the mortality profile (LT_{50} = 4 ± 0.2 days, 88% cumulative mortality by 9 days pi) mirrored that of the lower dose at 24°C (Fig. 1), indicating that the susceptibility of the larvae to *M. robertsii* is an order of magnitude lower at the cooler temperature.

Adhesion, germination and colonization assay

The adhesion of conidia to the larval cuticle was greater at the cooler temperature (24°C). Almost twice as many conidia adhered at the higher inoculum dose, and 1.3 times more at the low dose, compared with conidia at 34°C (Table 1; $F_{1,27} = 12.7$, $P = 0.002$).

The germination of conidia on the insect cuticle was minimal at 6 h pi, irrespective of the dose and temperature (all treatments ≤ 4.1% germination). However, by 24 h pi a higher proportion of the conidia germinated at the cooler temperature ($F_{1,29} = 3.77$, $P = 0.063$). The presence of the fungus in the hemolymph was only observable by 48 h pi in larvae inoculated with 10^8/mL conidia at 24°C ($\chi^2 = 32.7$, df = 1, $P < 0.0001$). None of the fungus-challenged insects at the warmer temperature showed evidence of colonization by this time point, and indeed larvae receiving the 10^7/mL dose at 34°C recovered from infection.

Epicuticular fatty acids and their effect on fungal germination

Four major fatty acid constituents were recovered from the cuticles of uninfected larvae after 24 h incubation at each chosen temperature: palmetic (C16), stearic (C18), oleic (C18:1), and linoleic (C18:2) acid (Fig. 2a). With the exception of stearic acid, the quantity of all the fatty acids was significantly elevated after 24 h incubation at the cooler 24°C temperature, relative to 34°C (4 to 7-fold; Z value > 1.96, $P < 0.05$).

The influence of these fatty acids on the germination of *M. robertsii* conidia was then assessed *in vitro* using a range of concentrations of synthetic analogues (0.01%–1%, with the lowest concentration (0.01%) being in the physiological range). Exposure to each of the three fatty acids resulted in a trend of dose-dependent suppression of germination: palmetic acid ($r = –0.97$, $P = 0.03$, $n = 4$), oleic acid ($r = –0.99$, $P = 0.01$, $n = 4$) and linoleic acid ($r = –0.99$, $P = 0.005$, $n = 4$; Fig. 2B). Germination was significantly compromised by the highest concentrations of oleic and linoleic acid (respectively, $P < 0.05$ and $P < 0.01$ compared with the controls without fatty acids; Fig. 2B).

Phenoloxidase activity

Major differences in the kinetics and magnitude of PO activity were observed in *G. mellonella* larvae maintained under the different temperatures. The basal (uninfected) level of integument PO activity at 34°C was 11-fold greater than at 24°C ($P < 0.01$; Fig. 3A,B). At the cooler temperature (24°C), fungus infection triggered a significant increase in integument PO activity, but only after a delay of 48 h ($P < 0.001$ for 10^8/mL and $P < 0.01$ for 10^7/mL versus controls), coinciding with fungal colonization of the hemocoel (as detailed in Table 1). In contrast, at 34°C PO activity was already significantly increased by 6 h pi ($P < 0.05$ for the 10^8/mL and $P < 0.01$ for 10^7/mL), coinciding with the

phase of conidial adhesion and the start of germination (Table 1). It is noteworthy that at 34°C, the longevity of the PO response was dependent on the fungal dose. Although the PO responses were initially similar for both doses, PO activity dropped significantly by 48 h pi in response to the lower dose (i.e. 10^7/mL; $P < 0.01$ relative to the uninfected control) whereas at the higher dose (10^8/mL) PO activity remained elevated (Fig. 3B).

Hemolymph PO activity under basal (uninfected) conditions at 24°C was 1.2-fold lower than at 34°C ($P < 0.05$; Fig. 3C,D). Relative to the uninfected control, hemolymph PO activity decreased at 6 h pi with both doses of the fungus at 24°C ($P < 0.01$ for 10^8/mL and $P < 0.01$ for 10^7/mL; Fig. 3C,D). Subsequently, only insects exposed to the lower inoculum dose exhibited a consistent increase in PO activity (significant by 48 h pi $P < 0.01$ compared with the control and $P < 0.001$ compared with insects exposed to the high dose). At the optimal temperature, (34°C) no significant changes in hemolymph PO activity were observed, irrespective of the infection status of the insects.

Encapsulation response

The *G. mellonella* basal (uninfected) encapsulation response was robust but not significantly affected by temperature (Fig. 3E,F). Furthermore, no significant changes in encapsulation activity occurred following fungal infection at either temperature, with the exception of insects exposed to the higher (10^8/mL) inoculum dose at 24°C. In this latter treatment group, a sharp decrease in encapsulation activity was observed 6 h pi ($P < 0.001$ at 24 and 48 h pi), which decreased to zero by 48 h pi, when the fungus was detected in the hemolymph.

Lysozyme-like activity

Basal lysozyme-like activity in the uninfected control larvae was 1.6 fold lower at the cooler temperature of 24°C compared with the optimal temperature of 34°C ($P < 0.001$; Fig. 3G,H). At 24°C, fungal infection had only a minor influence, with no significant changes in lysozyme-like activity in response to the higher dose (10^8/mL) even during invasion of the hemocoel at 48 h pi. A transient but significant dip in activity at 24 h pi in response to the lower dose was observed at both temperatures and was significant at 24°C (10^7/mL; $P < 0.05$; Fig. 3G,H). Following infection, lysozyme-like activity at the optimal temperature (34°C) remained elevated relative to 24°C, with minor fluctuations. At the optimal temperature, a small but significant increase occurred in insects infected with the higher inoculum dose at 24 and 48 h pi ($P < 0.05$; Fig. 3H). From 24 h higher lysozyme activity was observed at the higher dose, at both temperatures, which corresponded with increased germination rates.

Gallerimycin and galiomicin gene expression

Temperature and fungal infection both had dramatic impacts on the fat body expression of the AMP genes *gallerimycin* (*gal*) and *galiomicin* (*galio*). In uninfected insects, sustained exposure to the cool temperature (24°C) triggered greatly enhanced expression of both genes over 2 days, peaking after just 6 h (relative to expression at 34°C at 6 h: *gal* 591-fold, *galio* 295-fold; at 24 h: *Gal* 24-fold, *galio* 45-fold; at 48 h: *gal* 2.3-fold, *galio* 4.7-fold; Fig. 4).

In fungus-infected larvae, the expression of both AMP genes significantly increased in the three treatment groups in which the insects suffered high mortality. However, up-regulation beyond basal levels in these groups was delayed, only occurring at significant levels at 48 h pi (Fig. 5) for both genes, and with *galio* being the most highly expressed. Specifically, at the cool temperature (24°C), expression levels relative to the uninfected controls at 48 h pi were as follows: 914-fold for *gal*, 19453-fold for *galio* (10^7/mL treatment, $P < 0.001$), and 13972-fold for *gal*, 46324-fold for *galio* (10^8/mL treatment, $P < 0.01$) (Fig. 5A,B). The response to the 10^8/mL dose at 34°C was similar to that at the cooler temperature, but at 48 h pi orders of magnitude less powerful: 10-fold for *gal* and 511-fold for *galio* ($P < 0.05$) (Fig. 5B). In contrast, the expression of both AMP genes during the only survivable treatment (that of 10^7/mL conidia at 34°C) showed only minor fluctuations above and below basal expression, none of which was a significant change.

Discussion

The optimum temperature for the development of the wax moth corresponds to that of the brood chamber in beehives. However, most studies on this insect have been conducted at significantly lower room temperature, sometimes with brief thermal shock, with the assumption that the results reflect what may happen in the wild. The current study shows that changes in environmental temperature dramatically alter host-pathogen dynamics, which can profoundly influence the infection outcome. Thus, although a virulent strain of *M. robertsii* causes rapid and substantial insect mortality at room temperature, a ten-fold higher dose is required to achieve the same outcome at the higher, sustained, temperature of 34^{o}C. Although such elevated doses are unlikely to be

encountered by the insect in the wild, they are relevant in biocontrol situations during the application of EPFs.

A sustained warm temperature, such as that encountered in beehives, affects both the fungus and the insect host. In the current study it interfered with conidial adhesion and germination, and also retarded fungal development in the hemocoel, which allowed the host more time to mobilize its defenses to eliminate the pathogen. In warmer insects, immune defenses based on enzymes such as lysozyme and PO were elevated, even under basal (uninfected) conditions, presumably due to temperature-related enzyme kinetics. Similar effects on basal humoral immunity have been observed in *G. mellonella* by Wojda *et al*. (2009) following a short (30 min) exposure to 38^{o}C.

The relationship between the fatty acid composition of the insect epicuticular waxes and resistance was unclear. The fatty acid profile is in agreement with that described in *G. mellonella* by Gołębiowski *et al.* (2008). There were no major changes in the profile of insects kept at optimal (34^{o}C) and less optimal (24^{o}C) temperatures, but the relative quantities did increase for the latter. Although these fatty acids are known to inhibit a range of human and animal pathogenic fungi (e.g. Ment *et al*., 2013) and demonstrated fungistasis in our *in vitro* studies, this was not observed in insects kept at 24^{o}C. It is surprising that the relative amount of fatty acids decreased at the higher temperature, given that xerophilous arthropods produce high quantities of epicuticular lipids to prevent water loss. It is possible that the humidity was high enough in our experiments to obviate the physiological demand for enhanced fatty acid synthesis.

Although it is well established that EPF-induced mortality is dose-dependent, what is clear from this study is that 10^8 conidia/mL is the threshold dose beyond which no temperature-dependent physiological changes will protect the insect. Wax moth larvae are unlikely to encounter this dose in nature because growth of most EPF stops or is retarded at hive temperatures, and because hives are kept scrupulously clean by the worker bees. This may explain why the larvae in the current study did not exhibit a strong AMP response compared with insects kept at less optimal temperatures. The kinetics of the response in infected insects suggests that significantly enhanced AMP expression is triggered by fungal invasion of the hemocoel and not by some earlier event in the infection process. It is also notable that the magnitude of the response is in proportion to the relative number of conidia penetrations, and therefore appears to be only indirectly related to temperature. It also suggests a pharmacological response (i.e. fungal elicitor dependent response). Mowlds *et al*. (2010) also noted that the intensity of the defence response corresponded with the elicitor concentration (e.g. *β*-glucan).

The current study suggests that the infectivity and dose of the fungus are stronger determinants of morbidity and mortality in *G. mellonella* than its immune responses. The magnitude of the immune responses broadly correlated with the number of fungal propagules that invaded the insect, but the insects were nevertheless overwhelmed and killed. It is important to note that by eliminating competition from bacterial species, strong anti-pathogen responses may even indirectly benefit the fungus (Butt *et al*., 2016). Only the warmest insects inoculated with the lowest dose of conidia were able to withstand the infection, yet this group exhibited the lowest AMP gene expression. It is

unclear whether these modest increases in AMP gene expression were effective at combatting the fungus, or whether the fungus simply failed to mount a serious invasion of the hemocoel. The large but transient up-regulation of gallerimycin and galiomicin gene expression seen in uninfected larvae at the low temperature is most likely a non-specific cold shock stress response. Similar elevations of antimicrobial peptides in various insects under cold or heat exposure have been observed by others (e.g. Mowlds & Kavanagh, 2008; Wojda *et al.*, 2009; Xu & James, 2012; Wojda &Taszłow, 2013).

The relative speed of the integument PO responses is noteworthy. At 34^{o}C, but not at 24^{o}C, PO responses were already significantly above the baseline by 6 h pi with both fungal doses: a time point at which < 5% of the conidia had germinated in any of the experimental groups. This indicates a superior capacity to detect and respond locally to low levels of fungal antigens with an obvious candidate molecule being the fungal serine protease Pr1. The activity of this enzyme increases with temperature independent of germination and is known to activate the PO cascade (St Leger *et al*., 1996; Butt *et al*., 2013). The important role of cuticular PO in wax moth defense against the initial stages of mycoses has been highlighted previously by Dubovskiy *et al*. (2013a,b).

Encapsulation does not appear to be a major player in protecting the fungus at either low or high temperatures. The dramatic decrease in nylon implant encapsulation at 24^{o}C at the high dose presumably reflected a massive recruitment of hemocytes towards high numbers of invading fungal propagules, combined with the cytotoxic activities of fungal metabolites (Charnley, 2003).

Wax moths are much more susceptible to EPFs than bees (e.g. Mommaerts & Smagghe, 2011), and would be a good indicator of the EPF-related threats / risks to which bees are exposed in treated areas. The fact that commonly used EPFs cannot survive elevated temperatures is helpful in protecting human end-users, but it has implications for their use in glasshouses and hot-climate countries, as both their efficacy and speed of killing could be compromised. It is therefore important to select EPF strains with higher temperature tolerances, and to augment with agents that enhance their efficacy, such as oil formulations (Bateman *et al*., 1993; Santi *et al*., 2011). Our results indicate that with the correct strain, formulation, and with a sufficiently high dose, insect host defenses can be overcome even at elevated habitat temperatures.

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Disclosure

The authors declare that they have no conflict of interests.

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Fig. 1 The effect of environmental temperature and fungal dose on mortality of *Galleria mellonella* larvae after infection with *Metarhizium robertsii*. Larvae were topically infected with 10^7 or 10^8 conidia/mL and incubated for 9 days at either 34°C (optimal for the insect) or 24°C (optimal for the fungus). $^{\S}P < 0.01$ compared with control uninfected larvae at the same temperature. $^{\dagger}P < 0.01$ compared with larvae infected with the same dose but at the alternative temperature. $^{*}P < 0.01$ versus larvae infected with the alternative fungal dose at the same temperature.

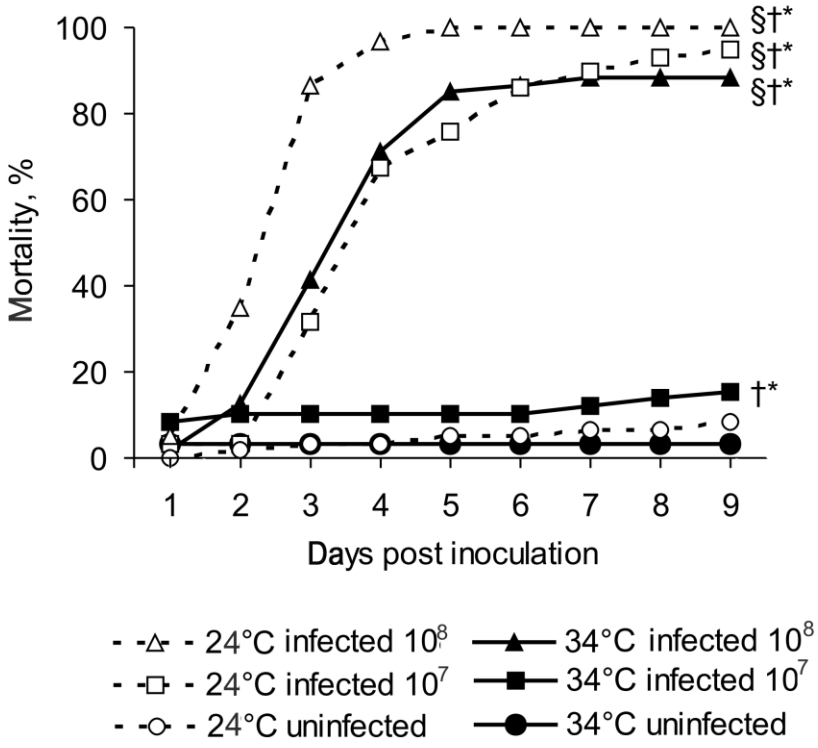

Fig. 2 Major *G. mellonella* cuticular fatty acids and their inhibitory influence on *M. robertsii* germination. (A) The quantity and identity of major fatty acids extracted from the epicuticle of uninfected larvae maintained for 24h at either 34°C or 24°C. Median and 25%–75% quartile deviation. *Z value > 1.96, P < 0.05. (B) The *in vitro* germination rate of *M. robertsii* conidia incubated for 24 h on agar supplemented with three concentrations of palmetic (C16), oleic (C18:1) and linoleic (C18:2) fatty acids. *P < 0.05, **P < 0.01 (inhibitory effects of C18:1 and C18:2 on germination compared with a control growth medium of n-hexane on agar).

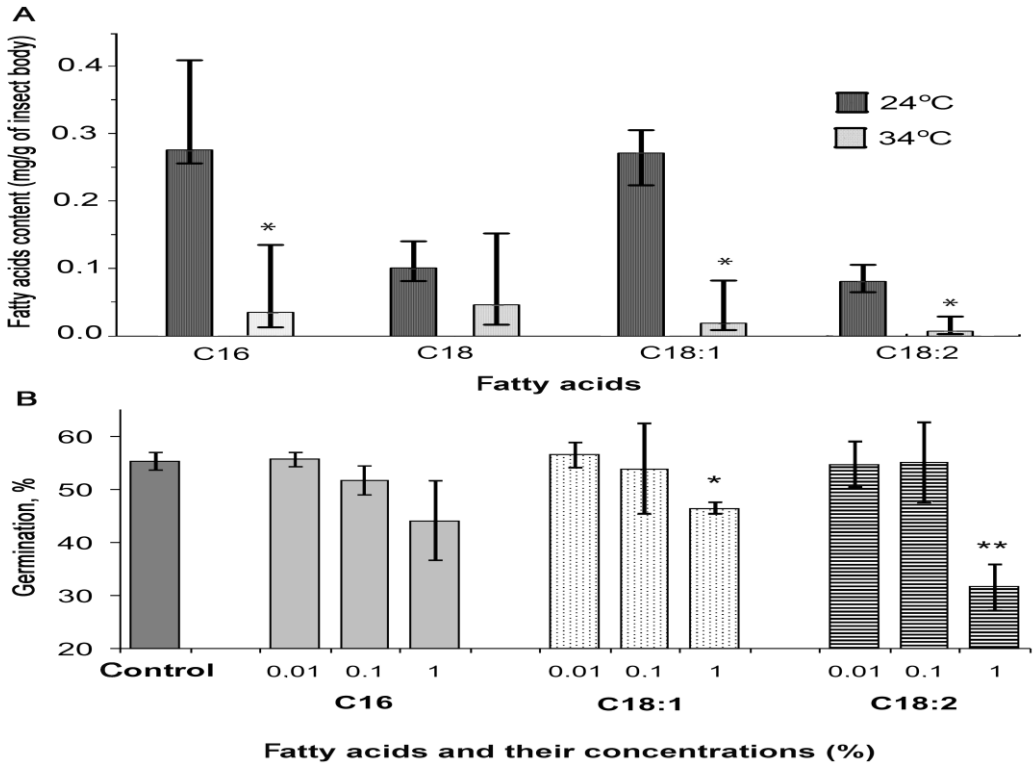

Fig. 3 Immune responses of *G. mellonella* larvae maintained at different temperatures following *M. robertsii* infection at two different doses. Larvae were topically infected with 10^7 or 10^8 conidia/mL and incubated for 48 h at either 34°C (optimal for the insect) or 24°C (optimal for the fungus). Cuticular phenoloxidase (PO) activity (A,B), hemolymph phenoloxidase (C,D), encapsulation responses (E,F), and lysozyme-like activity in hemolymph (G,H) of *G. mellonella* larvae following topical inoculation with *M. robertsii* (10^7/mL or 10^8/mL) at 24°C or 34°C. Data presented as the mean ± SEM; the height of the gray (control) bar represents the mean ± SEM. $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ compared with uninfected larvae at 24°C; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with uninfected control larvae with the same treatment; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with larvae infected with 10^8/mL dose of conidia at the same time point).

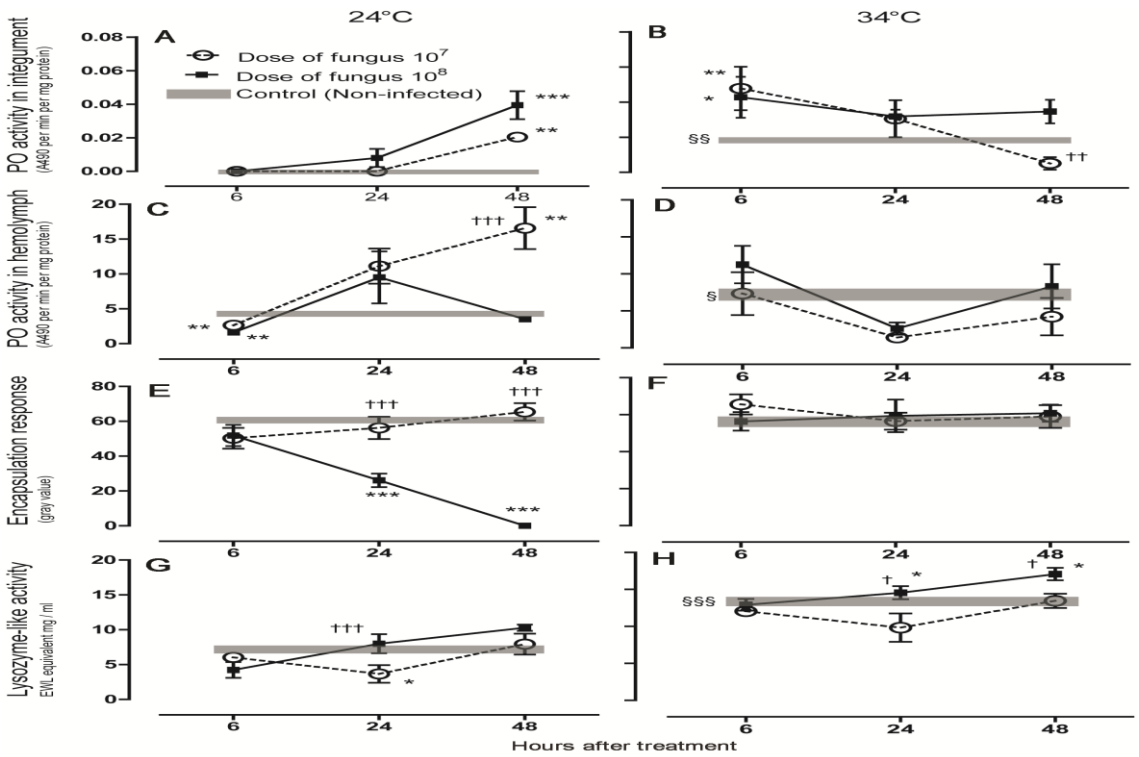

Fig. 4 The influence of environmental temperature on the basal expression of two AMP genes in the fat body of uninfected *G. mellonella* larvae. Fold changes are relative to uninfected larvae maintained at 34°C; *P < 0.05 compared with 6h time point. Data are presented as the mean ± SE and analyzed by one-way ANOVA (Kruskall-Wallis with Dunn's post test).

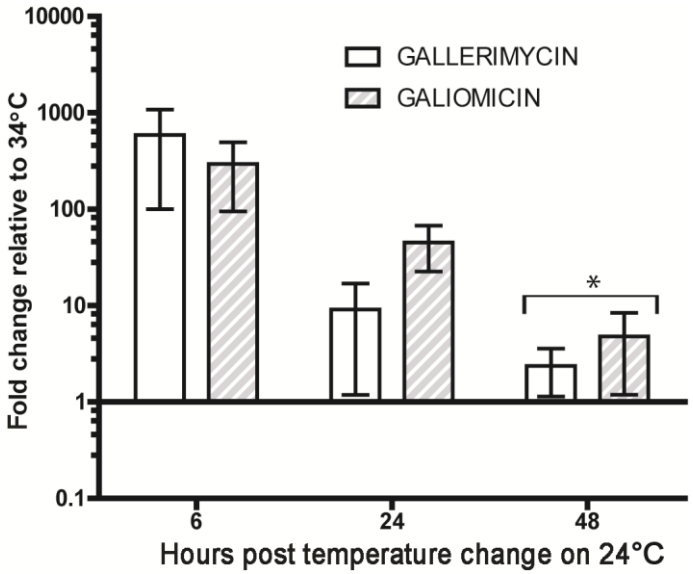

Fig. 5 The influence of temperature and fungal dose on the expression of two AMP genes in *G. mellonella* larvae infected with *M. robertsii*. *Galiomicin* and *gallerimycin* expression was measured in fat body following topical application of two different doses of *M. robertsii* (10^7/mL and 10^8/mL), in larvae maintained at either (A) 24°C or (B) 34°C. *Y*-axis shows the fold change relative to uninfected larvae maintained at the same temperature for the same time period. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with 6h time point for the same conditions. Data are presented as the mean ± SE and analyzed by one-way ANOVA (Kruskall-Wallis with Dunn's post test).

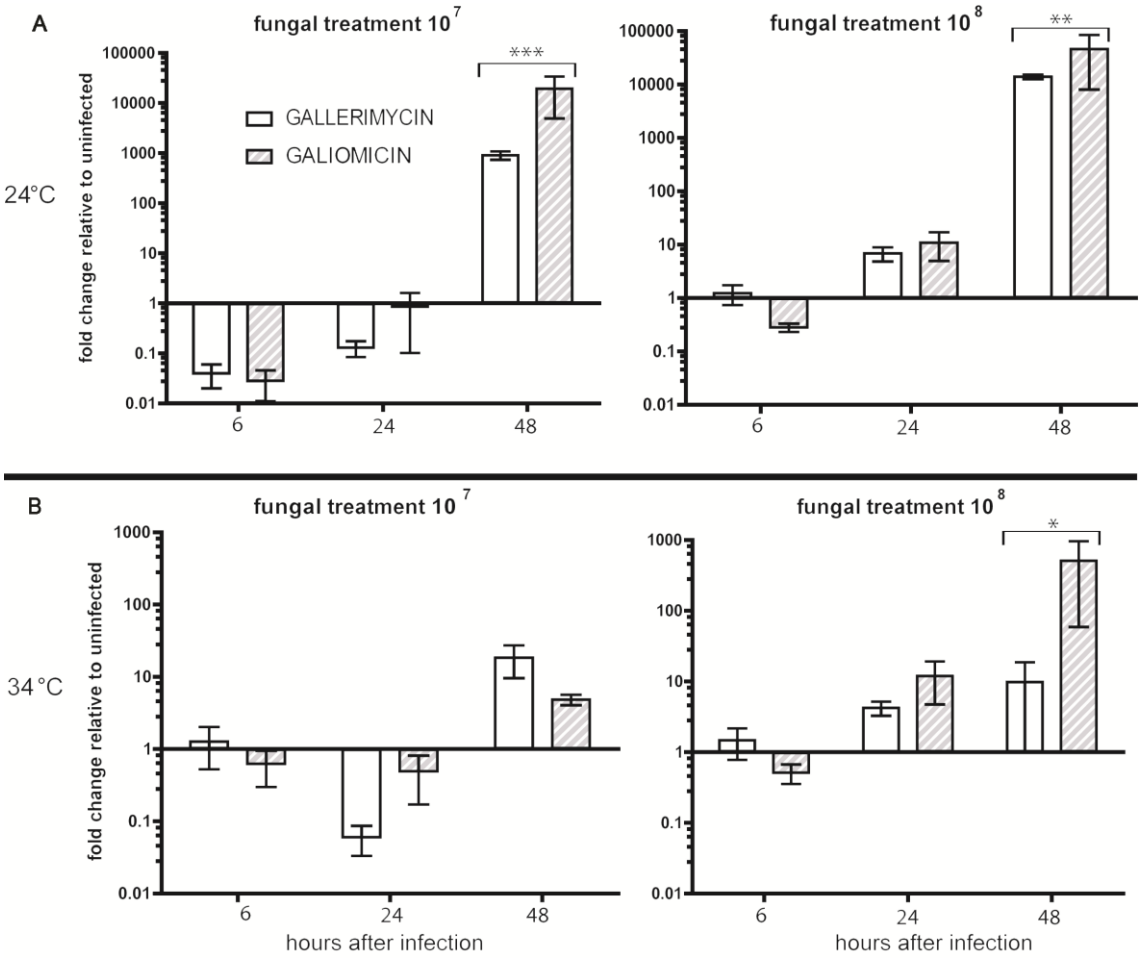

Table 1 The influence of environmental temperature and fungal dose on key developmental stages of *M. robertsii* following topical inoculation of *Galleria mellonella* larvae. Larvae were infected with 10^7 or 10^8 conidia/mL and incubated for 48 h at either 34°C or 24°C.

Concentrations (conidia/mL)	t°C	Adhered conidia / larva	% germinated conidia	% of colonized larvae		
		6 h	6 h	24 h	24 h	48 h
10^8	34	$1.2 \pm 0.2 \times 10^6$	3.8 ± 1.0	55.3 ± 14.3	nd	nd
	24	$2.4 \pm 0.3 \times 10^6$*	3.3 ± 1.4	80.6 ± 8.5	nd	80*
10^7	34	$1.4 \pm 0.3 \times 10^5$	2.3 ± 0.7	46.9 ± 15.4	nd	nd
	24	$1.9 \pm 0.2 \times 10^5$*	4.1 ± 1.8	60.6 ± 12.4	nd	nd
Effect of temperature	$F = 12.7$ $P = 0.0016$	$F = 0.66$ $P = 0.42$	$F = 3.77$ $P = 0.063$		$\chi^2 = 32.7$ $P < 0.0001$	

*$P < 0.01$ compared with the optimal temperature (34°C) at the same concentration of conidia; nd – hyphal bodies were not detected in larvae.