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Study of temperature–growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development

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ABSTRACT

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Aims: To investigate the thermal biology of entomopathogenic fungi being examined as potential microbial control agents of *Varroa destructor*, an ectoparasite of the European honey bee *Apis mellifera*.

Methods and Results: Colony extension rates were measured at three temperatures (20, 30 and 35°C) for 41 isolates of entomopathogenic fungi. All of the isolates grew at 20 and 30°C but only 11 isolates grew at 35°C. Twenty-two isolates were then selected on the basis of appreciable growth at 30–35°C (the temperature range found within honey bee colonies) and/or infectivity to *V. destructor*, and their colony extension rates were measured at 10 temperatures (12·5–35°C). This data were then fitted to Schoolfield *et al.* [J Theor Biol (1981)88:719–731] re-formulation of the Sharpe and DeMichele [J Theor Biol (1977)64:649–670] model of poikilotherm development. Overall, this model accounted for 87·6–93·9% of the data variance. Eleven isolates exhibited growth above 35°C. The optimum temperatures for extension rate ranged from 22·9 to 31·2°C. Only three isolates exhibited temperature optima above 30°C. The super-optimum temperatures (temperature above the optimum at which the colony extension rate was 10% of the maximum rate) ranged from 31·9 to 43·2°C.

Conclusions: The thermal requirements of the isolates examined against *V. destructor* are well matched to the temperatures in the broodless areas of honey bee colonies, and a proportion of isolates, should also be able to function within drone brood areas.

Significance and Impact of the Study: Potential exists for the control of *V. destructor* with entomopathogenic fungi in honey bee colonies. The methods employed in this study could be utilized in the selection of isolates for microbial control prior to screening for infectivity and could help in predicting the activity of a fungal control agent of *V. destructor* under fluctuating temperature conditions.

Keywords: fungi, microbial control, Schoolfield *et al.* model, Sharpe and DeMichele model, temperature, *Varroa destructor*, vegetative growth.

INTRODUCTION

The varroa mite, *Varroa destructor* is an important ectoparasite of the European honey bee *Apis mellifera* (Sammataro *et al.* 2000). *Varroa destructor* originated in Asia but has spread world-wide in recent years and is causing severe damage to *A. mellifera* populations. Adult female *V. destructor* feed on the haemolymph of adult bees and pupae and reproduction takes place within sealed brood. During feeding, mites can activate and transmit viral diseases which reduce the life expectancy of the bees and cause the colony

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to decline, particularly over winter (Ball 1994a,b). *Varroa destructor* has had a major impact in all countries where it has become established. The overwintering mortality of managed honey bee colonies in 1996, for example, was estimated to be equivalent to a quarter of the global commercial bee population (Sanford 1996).

At present, *V. destructor* is controlled with chemical pesticides, but resistance has already been recorded (Hillesheim *et al.* 1996; Thomas 1997; Elzen *et al.* 1998; Milani 1999; Anon. 2001) and so there is a need for alternative, sustainable forms of management, including biological control (Chandler *et al.* 2001). Entomopathogenic fungi are important natural enemies of Acari (Chandler *et al.* 2000) and have been shown to be virulent to *V. destructor* in laboratory experiments with negligible effect on honey bees (Shaw *et al.* 2002). In laboratory bioassays at 25°C/100% R.H., 40 isolates of fungi from six genera (*Verticillium*, *Hirsutella*, *Paecilomyces*, *Beauveria*, *Metarhizium* and *Tolypocladium*) were pathogenic to *V. destructor*, and some of these may be suitable for development as mycopesticides (Shaw *et al.* 2002). Low humidity, which is normally a major constraint to fungal infectivity, does not appear to be a limiting factor in this case (Shaw *et al.* 2002). However, the high temperatures maintained within some areas of the honey bee colony could have a significant impact on mycopesticide activity, because most isolates of entomopathogenic fungi require moderate temperatures (20–30°C) for optimum growth and development (Ferron 1978; Burges 1981; Fargues *et al.* 1997; Vidal *et al.* 1997).

The temperature relations within honey bee colonies are complex, as honey bees thermoregulate their colonies according to the season and the presence or absence of brood (Seeley and Heinrich 1981). *Varroa destructor* preferentially reproduces on drone brood, which is located on the periphery of the brood nest and is maintained at 32·5–33·4°C (Le Conte *et al.* 1990). Temperatures within broodless areas of the colony are around 25°C, but vary with ambient conditions (Simpson 1961). If ambient temperatures drop below 18°C, honey bees cluster together to reduce heat loss and generate warmth through increased metabolism (Simpson 1961; Seeley and Heinrich 1981; Winston 1987). In temperate regions, the temperature within a broodless winter cluster is normally 20–30°C, with a daily fluctuation of about 5°C (Free and Spencer-Booth 1959; Simpson 1961; Butler 1974; Winston 1987). The effectiveness of a cluster increases with its size, and a colony of at least 2000 bees is needed to survive low temperatures (Free and Spencer-Booth 1959; Southwick 1984). For this reason, colonies reduced in size by *V. destructor* and its associated viral diseases are particularly vulnerable to cold weather. A mycopesticide of *V. destructor* is therefore likely to be most valuable when used to control viral incidence in bee stocks prior to overwintering. This would probably entail applications in late summer, as applications in the autumn and winter run the risk of disturbing the honey bee cluster and are best avoided. A thorough understanding of the effects of high temperature on fungal development is therefore necessary to ascertain whether a mycopesticide can be used in the summer.

This paper describes investigations of the thermal biology of isolates of entomopathogenic fungi that are being examined as potential microbial control agents of *V. destructor*. The objective was to assess the ability of candidate isolates to operate under the temperature conditions in a honey bee colony. This information could be used then to inform the selection of isolates for microbial control. The effect of temperature on fungal development was studied by measuring colony extension on a solid medium at different temperatures. The data were analysed using Sharpe and DeMichele's (1977) mechanistic model of poikilotherm development [reparameterized by Schoolfield *et al.* (1981)].

MATERIALS AND METHODS

Fungal isolates

Forty-one isolates of fungi from six genera were used in the study: *Beauveria* (seven isolates), *Hirsutella* (11 isolates), *Metarhizium* (eight isolates), *Paecilomyces* (three isolates), *Tolypocladium* (two isolates) and *Verticillium* (10 isolates) (Table 1). Thirty-four of the isolates have been examined as potential microbial control agents of *V. destructor* (Shaw *et al.* 2002), whilst the remainder are awaiting assessment. Stock cultures of the isolates were stored in liquid nitrogen vapour (Chandler 1994). Laboratory cultures were grown on Sabouraud dextrose agar (SDA) slopes and maintained in a refrigerator at 4°C for up to 6 months. Subcultures for laboratory experiments were prepared on SDA from the slope cultures and incubated at 23 ± 1°C for 10–12 days in the dark. Conidia for colony extension experiments were harvested in sterile 0·05% Triton X-100 and enumerated using an improved Neubauer haemacytometer. Aliquots (10 ml) were prepared at a concentration of 1×10^7 ml^{-1}.

Effect of temperature on the colony extension of fungal isolates

Two experiments were performed to measure the effect of temperature on the colony extension of isolates. In experiment 1, the rates of colony extension of 41 isolates were measured at 20, 30 and 35°C. In experiment 2, 22 isolates were selected from experiment 1 on the basis of

appreciable growth at 35 or 30°C and/or infectivity to *V. destructor* (Shaw *et al.* 2002). The rates of colony extension of these isolates were then measured at 10 temperatures from 12·5 to 35°C. The method used to measure colony extension was the same for both experiments. For each isolate, 100 μl of conidial suspension was spread evenly over SDA (15 ml) in Petri dishes (90 mm diameter, triple vented) and incubated in the dark at 23°C for 48 h. Plugs (6 mm) cut from these cultures with a flame-sterilized cork borer were then placed upside down in the centre of fresh SDA (15 ml) in Petri dishes (90 mm diameter, triple vented), one plug per dish. The dishes were sealed in polyethylene bags and incubated for 28 days in darkness at the test temperature (incubator temperatures were monitored throughout using data loggers) with two dishes for each isolate × temperature combination. Colony diameters were measured with a ruler every 3–4 days for the duration of the experiment. Experiment 1 was repeated six times and experiment 2 was repeated three times.

Data analysis

The rate of increase of fungal colony radius r was used as an indicator of the specific biomass accumulation rate μ, according to $dr/dt = \mu w$ (Trinci 1971; Cooke and Whipps 1993), where w represents the width of the peripheral growth zone. On a defined medium, w is constant and unaffected by changes in temperature (Trinci 1971; Inch and Trinci 1987) and hence dr/dt can be used to investigate the effect of temperature on μ. Mean colony radius was plotted against time for each isolate, temperature and replicate, and colony extension rate was calculated during the linear phase (Fargues *et al.* 1992). Colony extension rates from experiment 1 were compared by ANOVA (Genstat 2000). In experiment 2, the colony extension rates were averaged across the three replicates and plotted against temperature. The resulting curves were described using the Schoolfield *et al.* (1981) re-formulation of the Sharpe and DeMichele (1977) model of poikilotherm development. This model was chosen because it has a physical basis and was developed to describe growth processes. The model is based on the hypothesis of Johnson and Lewin (1946) and is derived from the following assumptions: (1) at all temperatures, the development rate of the organism is determined by a single hypothetical rate-controlling enzyme, (2) development rate is proportional to the product of the concentration of the active enzyme and its rate constant and (3) the rate-controlling enzyme is reversibly inactivated at high and low temperatures, but a constant total concentration (active + inactive) is maintained independent

Table 1 Fungal isolates used in the study. All isolates were examined in experiment 1 and selected isolates only in experiment 2

Species	Isolate*	Host or source	Geographic origin	Experiment 2
Beauveria bassiana	322·89	*Hypothenemus hampei* (Coleoptera: Hyponomeutidae)	Brazil	
	431·99 (T228)[a]	Acari	Denmark	
	432·99[b]	*Anthonomus grandis* (Coleoptera: Curculionidae)	USA	✓
	433·99[c]	–	–	
	434·99 (ARSEF2869)[d]	*Bephratelloides cubensis* (Hymenoptera: Eurytomidae)	USA	✓
	454·99 (I91636)[e]	Acari	Oman	
	455·99 (DAT049)[f]	–	–	✓
Hirsutella sp.	435·99 (H1)[g]	Acari: Tarsonemidae	Poland	
	437·99 (H3)[g]	*Eriophyes piri* (Acari: Eriophyidae)	Poland	
Hirsutella kirchneri	46·81 (IMI251256)[e]	*Abacarus hystrix* (Acari: Eriophyidae)	UK	
	47·81 (IMI257456)[e]	*Abacarus hystrix* (Acari: Eriophyidae)	UK	
Hirsutella thompsonii	34·79	*Eriophyes guerreronis* (Acari: Eriophyidae)	Africa	✓
	51·81[h]	*Eriophyes guerreronis* (Acari: Eriophyidae)	Africa	✓
	71·82	*Eriophyes guerreronis* (Acari: Eriophyidae)	Jamaica	✓
	73·82[h]	*Phyllocoptruta captrila* (Acari: Eriophyidae)	USA	✓
	74·82[h]	*Eriophyes guerreronis* (Acari: Eriophyidae)	Africa	✓
	75·82[h]	*Colomerus novahebridensis* (Acari: Eriophyidae)	New Guinea	✓
	77·82[h]	*Eriophyes guerreronis* (Acari: Eriophyidae)	Jamaica	✓
Metarhizium anisopliae	441·99 (ARSEF3297)[d]	*Boophilus* sp. (Acari: Ixodidae)	Mexico	✓
	442·99 (ARSEF4556)[d]	*Boophilus* sp. (Acari: Ixodidae)	USA	✓
	443·99 (T248)[a]	Acari	Denmark	✓
	444·99 (ATCC38249)[i]	*Hylobius pales* (Coleoptera: Curculionidae)	–	✓
	445·99[j]	–	–	✓
	458·99[k]	–	–	
	459·99[l]	Acari	Israel	✓

Table 1 (*Contd.*)

Species	Isolate*	Host or source	Geographic origin	Experiment 2
Metarhizium flavoviride	203·84	*Nilaparvata lugens* (Hemiptera: Delphacidae)	Brazil	
Paecilomyces farinosus	446·99 (CCFC002085)[m]	*Mycobates* sp. (Acari: Mycobatidae)	Canada	
Paecilomyces fumosoroseus	409·96[n]	*Phenacoccus solani* (Hemiptera: Pseudococcisae)	–	
	447·99 (KVL319)[a]	*Ixodes ricinus* (Acari: Ixodidae)	Denmark	
Tolypocladium inflatum	448·99 (ARSEF3278)[d]	*Mycobates* sp. (Acari: Mycobatidae)	Canada	
Tolypocladium niveum	449·99 (CCFC002081)[m]	*Mycobates* sp. (Acari: Mycobatidae)	Canada	
Verticillium lecanii	1·72[o]	*Macrosiphoniella sanborni* (Homoptera: Aphididae)	UK	
	12·74 (IMI79606)[c]	*Chloropulvinaria floccifera* (Hemiptera: Coccidae)	Turkey	✓
	17·76	*Cecidophyopsis* sp. (Acari: Eriophyidae)	UK	✓
	19·79[p]	*Trialeurodes vaporariorum* (Homoptera: Aleyrodidae)	UK	✓
	30·79	*Cecidophyopsis* sp. (Acari: Eriophyidae)	UK	
	31·79	*Cecidophyopsis* sp. (Acari: Eriophyidae)	UK	
	450·99 (IMI235048)[c]	*Cecidophyopsis ribis* (Acari: Eriophyidae)	UK	✓
	451·99 (ARSEF1367)[d]	Acari: Orbatidae	Poland	
	452·99 (CBS317.70A)[q]	*Tetranychus urticae* (Acari: Tetranychidae)	–	✓
	453·99 (CCFC006079)[m]	Acari	Canada	✓

All isolates have been tested against *V. destructor* (Shaw *et al.* 2002), with the exception of *B. bassiana* 322·89, 454·99, 455·99, *M. anisopliae* 458·99, 459·99, *M. flavoviride* 203·84 and *V. lecanii* 12·74.

*Isolate number in the Horticulture Research International culture collection (isolate number from culture collection of origin).

[a]Kindly supplied by T. Steenberg, Danish Pest Infestation Laboratory, Skovbrynet 14, DK-2800, Lyngby, Denmark.

[b]Isolate forms the active ingredient in the proprietary mycopesticide 'BotaniGard' (Emerald BioAgriculture Corporation, 3125 Sovereign Dr, Lansing, MI 48911-4240).

[c]Isolate forms the active ingredient in the proprietary mycopesticide 'Naturalis' (Troy Biosciences Inc., 113 South 47th Avenue, Phoenix, AZ 850433, USA).

[d]Kindly supplied by the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF), USDA-ARS Plant Protection Research Unit, U.S. Plant, Soil and Nutrition Laboratory, Tower Road, Ithaca, NY, 14853-2901, USA.

[e]Obtained from the CAB International Mycological Institute, Bakeham Lane, Egham, Surrey, UK.

[f]Kindly supplied by Biocare Technology Pty Ltd, RMB 1084 Pacific Highway, Somersby, NSW 2250, Australia.

[g]Kindly supplied by C. Tkaczuk, University of Podlasie, Department of Plant Protection, ul. Prusa 14, 08110 Siedlce, Poland.

[h]Kindly supplied by C.W. McCoy, University of Florida, CREC, 700 Experiment Station Road, Lake Alfred, FL 33850, USA.

[i]Obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA, 20110-2209, USA.

[j]Isolate forms the active ingredient in the proprietary mycopesticide 'Bio-Blast' (Eco-Science Corporation, 17 Christopher Way, Eatontown, NJ 07724, USA).

[k]Isolate forms the active ingredient in the proprietary mycopesticide 'Bio1020' (Bayer AG, Werk Leverkusen, 51368 Leverkusen, Germany).

[l]Kindly supplied by M. Samish, Division of Parasitology, Kimron Veterinary Institute, Bet Dagan 50250, PO Box 12, Israel.

[m]Obtained from the Canadian Collection of Fungal Cultures, ECORC Room 1015, K.W. Neatby building, C.E.F. Ottawa, Ontario, Canada.

[n]Isolate forms the active ingredient in the proprietary mycopesticide 'PFR97' (Thermo-Trilogy Corporation, 9145 Guildford Road, Suite 175, Columbia, MD 21046, USA).

[o]Isolate forms the active ingredient in the proprietary mycopesticide 'Vertalec' (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands).

[p]Isolate forms the active ingredient in the proprietary mycopesticide 'Mycotal' (Koppert Biological Systems, PO Box 155, 2650 AD Berkel en Rodenrijs, The Netherlands).

[q]Obtained from the Centraalbureau voor Schimmelcultures, PO Box 273, 3740AG, Baarn, The Netherlands.

of temperature. The model consists of a thermodynamic description of the rate-limiting enzymic reaction using Eyring's (1935) theory combined with terms to describe the inhibitory effects of high and low temperatures (Johnson and Lewin 1946; Hultin 1955). Although the original nomenclature is retained, no attempt is made to interpret the coefficients in terms of the reaction processes that determine fungal growth.

The Schoolfield *et al.* (1981) model was modified by us to accommodate higher optimum temperatures (303 K: equivalent to 30°C) than the original (298 K: equivalent to 25°C). Following inspection of Arrhenius plots, the model was modified further to exclude low temperature inactivation and to include some coefficients which were specific to isolate *i*. All coefficients were species-specific. The model for any isolate of a given species that we used is as follows:

$$r_i(T) = \frac{\rho_{i(303\,\mathrm{K})}\frac{T}{303}\exp\left[\frac{\Delta H_A^{\neq}}{\mathrm{R}}\left(\frac{1}{303}-\frac{1}{T}\right)\right]}{1+\exp\left[\frac{\Delta H_{H_i}}{\mathrm{R}}\left(\frac{1}{T_{1/2_{H_i}}}-\frac{1}{T}\right)\right]} \qquad (1)$$

where $r_i(T)$ is the growth rate of isolate i at temperature T(K), $\rho_{i(303\ \mathrm{K})}$ is the development rate of isolate i at the standard reference temperature of 303 K assuming no enzyme inactivation, R is the universal gas constant (8·314 J K^{-1} mol^{-1}), $\Delta H_A^{\neq}$ is the (species-specific) enthalpy of activation of the reaction that is catalysed by the enzyme (J mol^{-1}), ΔH_{H_i} is the change in enthalpy associated with high temperature inactivation of the enzyme (J mol^{-1}) in isolate i, $T_{1/2_{H_i}}$ is the temperature (K) at which the enzyme is 1/2 active and 1/2 high temperature inactive in isolate i.

A non-linear model like eqn (1) needs accurate starting values for the model-fitting procedure to converge. Starting values were required for the following parameters: $\Delta H_A^{\neq}$, $\rho_{i(303\ \mathrm{K})}$, ΔH_{H_i} and $T_{1/2_{H_i}}$. These starting values were obtained from Arrhenius plots as recommended by Schoolfield *et al.* (1981). Figure 1 illustrates how the Schoolfield *et al.* (1981) recommendation for choosing parameter starting values was implemented for an individual isolate. First of all a 'best fit' curve, line A, was drawn by eye. The linear portion of curve A was then extended towards the vertical axis (line B). Line B represents the behaviour of the isolate under the assumption of no enzyme inactivation and has a slope of $-\Delta H_A^{\neq}/\mathrm{R}$. Next, a vertical line was drawn corresponding to a temperature of 303 K (30°C). This line intersects line B at $\rho_{i(303\ \mathrm{K})}$, the rate of development at 303 K. The slope of curve A at the upper temperature range is approx. $(\Delta H_{H_i} - \Delta H_A^{\neq})/\mathrm{R}$. The most important starting value is $T_{1/2_{H_i}}$, the temperature (K) at which the enzyme is 1/2 active and 1/2 high-temperature inactive. To calculate this, line C was drawn with values one half of line B, obtained by subtracting $\log_e 2$ from line B. $T_{1/2_{H_i}}$ corresponds to the point of intersection of lines A and C.

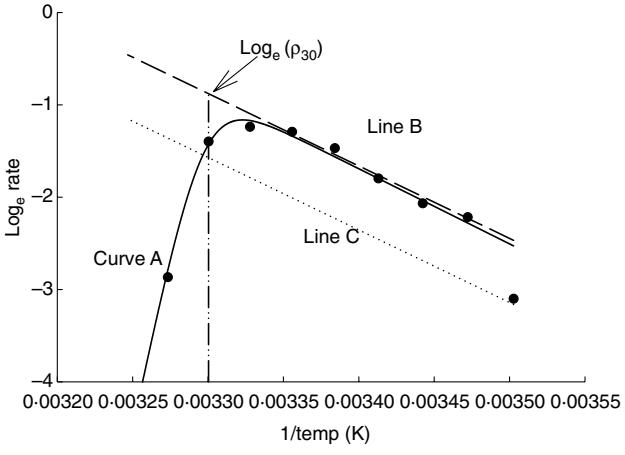

Fig. 1 Arrhenius plot of colony extension rate of *Hirsutella thompsonii* (75·82) on SDA. ● Indicates observed mean extension rates at 10 different temperatures (no detectable growth at 35°C). The derivations of the lines are described in the text. For the purposes of this presentation, the 'best fit' curve (line A) has been drawn using a computer graphics package, but in normal practice it would be drawn by hand

The model was fitted simultaneously to all isolates of a species so that a common value of $\Delta H_A^{\neq}$ could be established. Maximum likelihood estimation was performed using the FITNONLINEAR command of Genstat (Genstat 2000) with starting values read from the Arrhenius plots (Schoolfield *et al.* 1981). The temperature response of each isolate was summarized by its maximum achievable colony extension rate, its optimum temperature and its super-optimum temperature. The optimum temperature was the temperature of the maximum colony extension rate. The super-optimum temperature was defined as the temperature above the optimum at which the colony extension rate was 10% of the maximum rate. These temperatures were estimated by function minimization in Genstat (Genstat 2000). All calculations were performed using the thermodynamic temperature (K), but for ease of interpretation, optimum and super-optimum temperatures are reported as Celsius temperature ($\theta_C/°C = T/K - 273$).

RESULTS

Experiment 1: measurement of rates of colony extension at 20, 30 and 35°C.

All the fungal isolates grew at 20 and 30°C (Table 2). Colony extension rates at 20°C varied from 0·17 mm day^{-1} (*Hirsutella thompsonii* 75·82) to 2·28 mm day^{-1} (*Metarhizium anisopliae* 445·99). Colony extension rates at 30°C varied from 0·02 mm day^{-1} (*Hirsutella kirchneri* 46·81) to 1·72 mm day^{-1} (*M. anisopliae* 445·99). The slowest growing fungi at 20 and 30°C tended to be isolates of *Hirsutella* spp. Only 11 fungal isolates grew at 35°C. Of these, seven isolates were *Hirsutella* spp., three were *V. lecanii* and one was *M. anisopliae*.

Experiment 2: modelling the colony extension response of selected isolates at 12·5–35°C

In the Arrhenius plots, slopes below the optimum were similar for all the isolates (data not shown) and although there was evidence of low temperature inactivation for some isolates, there were insufficient data to estimate low temperature parameters. This was not unexpected, because most of the isolates studied in this experiment were pre-selected for their ability to grow at the higher temperature range. Equation (1) was formulated therefore to exclude low temperature inactivation as described previously.

Table 2 Extension rates of 41 isolates of entomopathogenic fungi at 20, 30 and 35 °C. Data points are the mean extension rates of six replicates. Isolates with no detectable growth were omitted from the analysis. Numbers in parenthesis represent their rank at the temperature examined

Species	Isolate	Rate at 20°C (mm day^{-1})	Rate at 30°C (mm day^{-1})	Rate at 35°C (mm day^{-1})
Beauveria bassiana	322·99	1·30 (6)	1·06 (12)	0·04 (14)
	431·99	1·25 (8)	0·94 (17)	0·00 (22)
	432·99	0·63 (30)	1·26 (3)	0·02 (20)
	433·99	1·18 (11)	1·07 (11)	0·05 (13)
	434·99	1·25 (8)	1·13 (8)	0·04 (14)
	454·99	0·81 (28)	0·62 (28)	0·09 (12)
	455·99	1·11 (18)	1·13 (8)	0·00 (22)
Hirsutella sp.	435·99	0·38 (37)	0·30 (33)	0·00 (22)
	437·99	0·67 (30)	0·93 (18)	0·24 (8)
Hirsutella kirchneri	46·81	0·24 (40)	0·02 (41)	0·03 (16)
	47·81	0·34 (38)	0·18 (37)	0·03 (16)
Hirsutella thompsonii	34·79	0·32 (39)	0·70 (27)	0·18 (11)
	51·81	0·56 (32)	0·93 (18)	0·25 (7)
	71·82	0·52 (33)	0·88 (20)	0·66 (2)
	73·82	0·47 (35)	0·83 (24)	0·41 (3)
	74·82	0·51 (34)	0·85 (22)	0·20 (10)
	75·82	0·17 (41)	0·25 (35)	0·00 (22)
	77·82	0·43 (36)	0·79 (25)	0·34 (4)
Metarhizium anisopliae	441·99	1·11 (18)	1·11 (10)	0·03 (16)
	442·99	1·08 (20)	1·18 (6)	0·00 (22)
	443·99	1·13 (16)	0·99 (13)	0·00 (22)
	444·99	1·42 (3)	1·19 (5)	0·00 (22)
	445·99	2·28 (1)	1·72 (1)	0·23 (9)
	458·99	1·61 (2)	0·99 (13)	0·00 (22)
	459·99	1·29 (7)	1·20 (4)	0·02 (20)
Metarhizium flavoviride	203·84	0·79 (29)	0·74 (26)	0·00 (22)
Paecilomyces farinosus	446·99	1·12 (17)	0·06 (40)	0·00 (22)
Paecilomyces fumosoroseus	409·96	1·34 (5)	0·95 (16)	0·03 (16)
	447·99	1·24 (10)	0·14 (39)	0·00 (22)
Tolypocladium inflatum	448·99	1·15 (15)	0·87 (21)	0·00 (22)
Tolypocladium niveum	449·99	1·04 (23)	0·84 (23)	0·00 (22)
Verticillium lecanii	1·72	1·16 (12)	0·18 (37)	0·00 (22)
	12·74	1·16 (12)	1·14 (7)	0·26 (6)
	17·76	1·01 (25)	0·34 (30)	0·00 (22)
	19·79	1·16 (12)	0·20 (36)	0·00 (22)
	30·79	0·99 (27)	0·30 (33)	0·00 (22)
	31·79	1·08 (20)	0·33 (31)	0·00 (22)
	450·99	1·06 (22)	0·32 (32)	0·00 (22)
	451·99	1·04 (23)	0·59 (29)	0·00 (22)
	452·99	1·00 (26)	0·97 (15)	0·34 (4)
	453·99	1·35 (4)	1·29 (2)	0·78 (1)
LSD (196 df)		0·210	0·190	0·058

The interaction between temperature and fungal colony extension conformed closely to eqn (1). Overall, 87·6–93·9% of data variance could be accounted for by this model. The standard errors (S.E.s) of the mean colony extension rates were less than 10% of the mean rates in 80% of cases. Because of this high degree of precision, S.E.s were omitted from all graphs and statistical analyses were based on unweighted mean rates.

The temperature responses of all the isolates were asymmetric, with rapid inactivation at temperatures above the optimum, the rate of which differed amongst isolates (Fig. 2). When Arrhenius plots were drawn for all isolates (not shown), it was apparent that line B had a very similar slope for all the isolates of a species. Hence it was appropriate to constrain $\Delta H_A^{\neq}$ to be the same for all isolates of a species. Thus starting values for $\Delta H_A^{\neq}$ were estimated by eye from Arrhenius plots corresponding to Fig. 1.

The species-specific temperature response, $\Delta H_A^{\neq}$, indicated that, amongst the isolates tested, isolates of *H. thompsonii* were more sensitive to sub-optimal

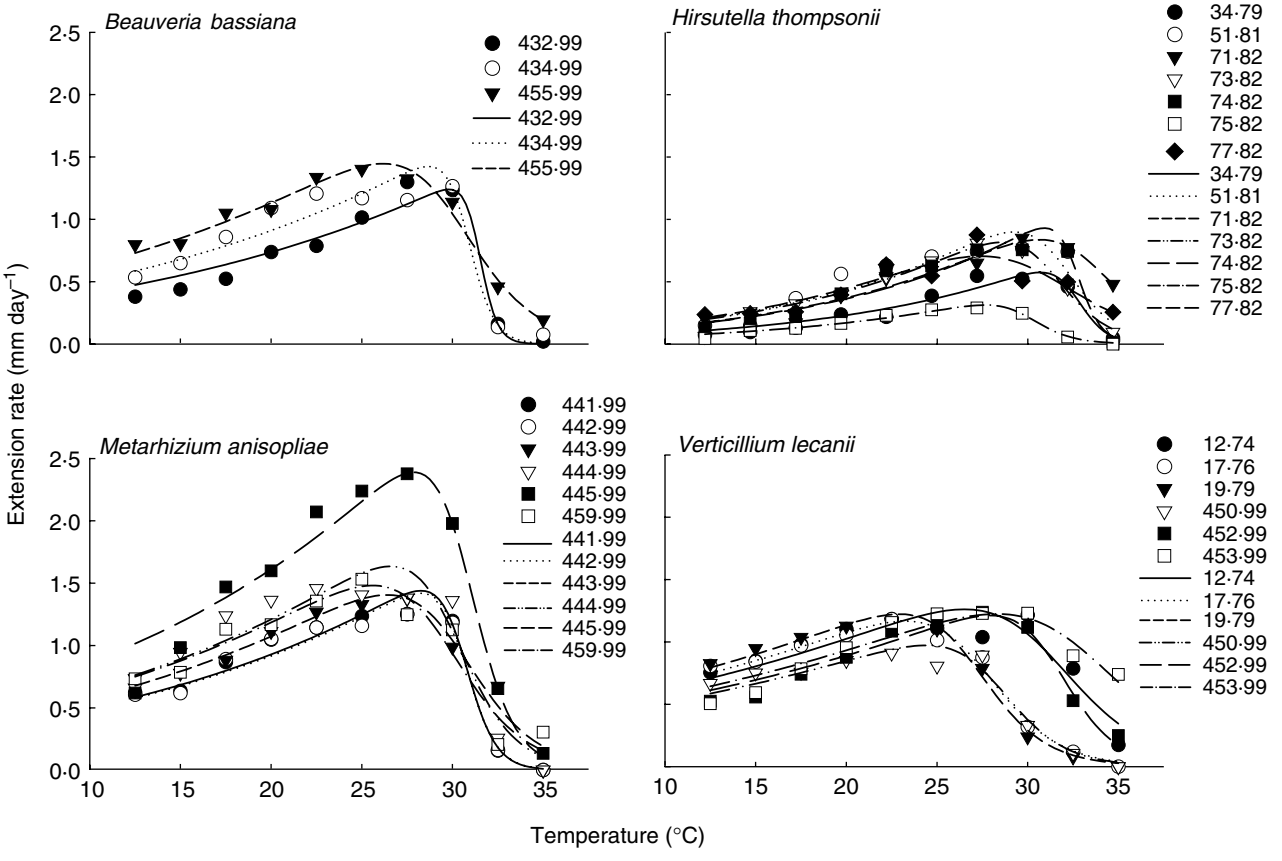

Fig. 2 Model predictions for the effect of temperature on the extension rate of colonies of entomopathogenic fungi (22 isolates from four genera). Data points are the mean extension rate of three replicates. Lines represent fitted curves obtained using equation (1). Isolate code numbers are listed in Table 1

temperatures than isolates of *B. bassiana*, *M. anisopliae* and *V. lecanii* (Table 3). Although the parameter estimates presented in Table 3 are useful for fitting further models, and possibly for classifying new isolates, they are difficult to interpret in terms of fungal biology. Table 4 provides a summary that can be related to fungal thermal biology. The optimum temperatures for the isolates ranged from 22·9°C (*V. lecanii* 19·79) to 31·1°C (*H. thompsonii* 34·79) (Table 4). Only three isolates exhibited temperature optima above 30°C and these were all *H. thompsonii* (isolates 34·79, 71·82 and 74·82) originating from the cassava green mite, *Eriophyes guerreronis*, in Africa. The super-optimum temperatures for the isolates ranged from 31·9°C (*V. lecanii* 19·79) to 43·2°C (*V. lecanii* 453·99). Super-optimum temperatures above 40°C were observed for two isolates (*H. thompsonii* 71·82 and *V. lecanii* 453·99) and super-optimum temperatures above 35°C were observed for 11 isolates (*B. bassiana* 455·99; *H. thompsonii* 34·79, 51·81, 71·82, 73·82 and 77·82; *M. anisopliae* 443·99 and 459·99; *V. lecanii* 12·74, 452·99 and 453·99).

DISCUSSION

Self-evidently, a fungal control agent of *V. destructor* will have to function under the physical conditions of a honey bee colony (Chandler *et al.* 2001). This paper was concerned primarily with measuring the response of fungal isolates to the high temperatures that are likely to be encountered in the brood nest area of honey bee colonies. The colony extension technique employed here is used widely to estimate the cardinal temperatures for fungal growth and is a valuable tool for screening entomopathogenic fungi prior to studies of their thermal requirements for infectivity (Fargues *et al.* 1992; Mietkiewski *et al.* 1994; Vidal *et al.* 1997). The response of fungi to temperature is a characteristic bell-shaped curve, skewed to the lower temperatures (Xu 1996). However, non-linear approaches to analysing entomopathogenic fungus–temperature interactions are not yet used routinely. In invertebrate mycopathology, the Sharpe and DeMichele (1977) model of poikilotherm development has been used to analyse the effect of

Table 3 Parameter estimates for the effect of temperature on the extension rates of colonies of entomopathogenic fungi (22 isolates from four genera). Estimations were based on the modified Sharpe and DeMichele (1977) model described in the text

Species	Isolate	$\Delta H_A^{\neq}$ (kJ mol^{-1})	$\rho_{(30°C)}$ (mm day^{-1})	ΔH_{Hi} (kJ mol^{-1})	$T_{1/2H}$ (K)
Beauveria bassiana	432·99	38·6	1·28	1650	304·5
	434·99	38·6	1·59	1135	303·9
	455·99	38·6	1·98	410	303·2
Hirsutella thompsonii	34·79	65·3	0·56	1027	306·0
	51·81	65·3	1·05	601	305·0
	71·82	65·3	0·90	462	306·9
	73·82	65·3	1·03	568	304·5
	74·82	65·3	0·88	1227	306·0
	75·82	65·3	0·42	715	303·3
	77·82	65·3	1·10	324	303·8
Metarhizium anisopliae	441·99	40·5	1·66	961	303·7
	442·99	40·5	1·64	955	303·7
	443·99	40·5	1·92	417	303·3
	444·99	40·5	2·17	516	303·1
	445·99	40·5	2·88	669	303·9
	459·99	40·5	2·13	404	302·6
Verticillium lecanii	12·74	32·3	1·65	342	304·3
	17·76	32·3	1·74	412	300·5
	19·79	32·3	1·85	438	300·1
	450·99	32·3	1·36	464	301·4
	452·99	32·3	1·43	535	304·7
	453·99	32·3	1·50	297	306·5

Table 4 Temperature responses of entomopathogenic fungi (22 isolates from four genera). Values are the estimates obtained using the modified Sharpe and DeMichele (1977) model described in the text

Species	Isolate	Optimum temperature (°C)	Super-optimum temperature (°C)	Rate at optimum temperature (mm day^{-1})
Beauveria bassiana	432·99	29·8	32·6	1·24
	434·99	28·7	32·6	1·42
	455·99	26·2	35·6	1·45
Hirsutella thompsonii	34·79	31·1	35·6	0·57
	51·81	29·6	36·0	0·90
	71·82	30·9	41·0	0·84
	73·82	28·8	35·6	0·82
	74·82	31·2	34·6	0·93
	75·82	28·0	33·4	0·31
	77·82	27·7	39·6	0·70
Metarhizium anisopliae	441·99	28·3	32·7	1·44
	442·99	28·3	32·7	1·42
	443·99	26·4	35·7	1·40
	444·99	26·6	34·2	1·63
	445·99	27·9	33·9	2·39
	459·99	25·6	35·1	1·48
Verticillium lecanii	12·74	25·8	38·7	1·23
	17·76	23·3	32·7	1·16
	19·79	22·9	31·9	1·22
	450·99	24·4	32·9	0·97
	452·99	27·9	35·6	1·21
	453·99	28·3	43·2	1·22

temperature on the mortality rate of twospotted spider mites, *Tetranychus urticae*, infected with the entomophthoralean fungus *Neozygites floridana* (Smitley *et al.* 1986), and to predict the rate of development of *Lagenidium giganteum* (Chromista, Oomycetes) in its mosquito host (Patel *et al.* 1991). A non-linear regression procedure (in SPSS for

Windows 6.1; SPSS, Inc., Chicago, IL, USA) has also been used to estimate the optimum temperature for colony extension of *M. flavoviride* (*M. anisopliae* var. *acridum*) isolates used as microbial control agents of locusts and grasshoppers (Thomas and Jenkins 1997).

The thermal requirements of the fungal isolates being examined against *V. destructor* are well matched to the temperatures in the broodless areas of honey bee colonies in temperate regions in the summer (*ca.* 25°C) (Simpson 1961). Our results indicate that a proportion of the isolates will also be able to function under the temperature conditions within drone brood areas on the periphery of the brood nest, where mites preferentially reproduce (32·5–33·4°C) (Le Conte *et al.* 1990). However, there are doubts about the ability of these isolates to operate in central areas of the brood nest where temperatures range from 33 to 36°C (Southwick and Heldmaier 1987). In general, isolates of *H. thompsonii* showed the best growth responses to high temperatures, although some isolates from the other species compared favourably, for example *V. lecanii* 453·99. In all cases, however, small increases in temperature above the optimum had a disproportionate effect on fungal development. With this in mind, the most valuable isolates are likely to be those not only with high temperature optima, but also with small values of $(\Delta H_{H_i} - \Delta H_A^{\neq})$ (i.e. shallow slopes at the upper temperature range). Our studies have focused on honey bee colony conditions in the summer, but the results show also that temperature conditions within winter colonies are unlikely to impair fungal activity.

There is merit in widening the search for isolates that are capable of killing *V. destructor* at temperature conditions in the centre of the brood nest. Of the fungal isolates examined in our study, those of *Hirsutella* spp. exhibited the most favourable temperature profiles, although other genera should also be included in future screening programmes. For example, an isolate of *M. flavoviride*, originating from Madagascar, grew best at 34°C and had a maximum temperature for growth of 38°C (Welling *et al.* 1994), while isolates of *M. anisopliae* have been described that germinated rapidly at 37°C (McCammon and Rath 1994) or grew at 40°C (Hallsworth and Magan 1999). The relationship between geographic origin and thermal biology did not form part of this study, however, entomopathogenic fungi exhibit adaptations to their thermal environments, which become more apparent towards the upper and lower thresholds (Fargues *et al.* 1992; Thomas and Jenkins 1997), and hence high temperature isolates are more likely to be found in hot environments. Isolates of *Paecilomyces fumosoroseus* from the tropics or subtropics, for example, tolerated high (32 and 35°C) temperatures better than isolates from European temperate zones (Vidal *et al.* 1997). Conversely, in Canada, cold active isolates of *M. anisopliae* were associated with more northerly latitudes (Amritha De Croos and Bidochka 1999).

The methods described in this paper provide valuable information on the thermal biology of fungi being tested against *V. destructor*. The Schoolfield *et al.* (1981) re-formulation of the Sharpe and DeMichele (1977) model described the data well and undoubtedly has wider applications in mycology. Xu (1996) has emphasized the importance of non-linear models in understanding and predicting fungal development at fluctuating temperatures, and in particular, the phenomenon whereby the development rate at fluctuating temperatures differs from the rate at a constant temperature, corresponding to the average of the fluctuating temperatures (which can be explained simply by the mathematical properties of nonlinear functions, the so-called Kauffman effect). For our work, the model could possibly help in predicting the activity of a microbial control agent against *V. destructor* at the fluctuating temperatures that would be experienced by a fungus carried in different areas of a colony. However, further research is required to determine the effects of temperature on fungal infectivity to *V. destructor*, and the persistence of conidia within honey bee colonies. Screening isolates for favourable temperature responses should now be easier, as the use of a species-specific coefficient $\Delta H_A^{\neq}$ enables thermal behaviour to be described fully, using only a small number of temperatures around the optimum.

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