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The inhibitory effect of the fungal toxin, destruxin A, on behavioural fever in the desert locust

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Abstract

During an infection locusts behaviourally **fever** [present] by seeking out higher environmental temperatures. This behaviour **places** [present] the pathogen at sub-optimal growth temperatures while improving the efficiency of the immune system, thereby prolonging the lifespan of the host. It **is** [present] therefore in the interest of the pathogen to either adapt to fever-like temperatures or to evolve mechanisms to interfere with, or inhibit fever. We **investigated** [past] the behavioural fever response of desert locusts to two fungal pathogens. A prolonged fever **was observed** [past] in locusts infected with *Metarhizium acridum*. However, fever **was** [past] comparatively shortlived during infection with *Metarhizium robertsii*. In both cases, restriction of thermoregulation **reduced** [past] lifespan. Destruxin A (dtx A) produced by *M. robertsii*, but not *M. acridum*, **has** previously **been associated** [present perfect] with the inhibition of the insect immune system. Injection of dtx A during infection with the fever causing *M. acridum* **inhibited** [past] fever and **was** [past] particularly effective when administered early on in infection. Furthermore, locusts **injected** [past] with dtx A **were** [past] more susceptible to *M. acridum* infection. Therefore, engineering *M. acridum* isolates currently used for locust biocontrol to express dtx A may **improve** [present] efficiency of control by interfering with fever.

[Note that the structure of the abstract resembles that of a pared-down paper]

1. Introduction

Isolates of the fungal entomopathogen *Metarhizium* spp. **have been developed** [present perfect] as biopesticides against a range of insects as an alternative to chemical pesticides. A formulation of the entomopathogen *Metarhizium acridum* (IMI330189) **has been developed** [present perfect] successfully for use against the desert locust, *Schistocerca gregaria* (Bateman et al., 1996; Langewald et al., 1997). During a fungal infection desert locusts behaviourally **fever** [present] by seeking out higher environmental temperatures than their healthy conspecifics (Bundey et al., 2003; Elliot et al., 2002). The temperatures achieved **are** [present] suboptimal for pathogen growth (Arthurs and Thomas, 2001; Blanford and Thomas, 2001) and **enhance** [present] other aspects of the immune response n.b. behavioural fever **is** [present] itself a component of immune defence (Ouedraogo et al., 2002). The survival advantage provided by behavioural fever **is** [present] thought to be responsible largely for variable speeds of kill by mycoinsecticides in the field (Blanford et al., 1998; Lomer et al., 2001). Thus one way of improving the efficacy of fungal biocontrol may **be** [present] to identify ways of interfering with the fever response.

Behavioural fever **has been reported** [present perfect] in a range of insects including Dictyoptera (Bronstein and Conner, 1984), Hymenoptera (Starks et al., 2000; Campbell et al., 2010), Diptera (Watson et al., 1993; Kalsbeek et al., 2001), Coleoptera (McClain et al., 1988) and Lepidoptera (Karban, 1998), though it **has been** best **characterized** [present perfect] in Orthoptera (Adamo, 1998; Blanford et al., 1998; Blanford and Thomas, 1999; Elliot et al., 2002; Bundey et al., 2003). Furthermore, behavioural fever **has** also **been reported** [present perfect] in vertebrates suggesting a conserved evolutionary ancestry (Blatteis and Smith, 1980; Kluger, 1991; Florez-Duquet et al., 2001). Unlike the regulatory mechanisms of physiological fever in mammals, the pathways involved in behavioural fever **are** [present] largely unknown. Evidence from injecting locusts with inhibitory chemicals of the same pathways does however **indicate** [present] similar mechanisms **have been conserved** [present perfect] (Bundey et al., 2003). Toxic secondary metabolites, of which the

destruxins (dtxs), a family of cyclic depsipeptides, are the most abundant, **have been identified** [present perfect] from a number of *Metarhizium* spp. isolates. These toxins **have** [present] a wide variety of effects *in vitro* (for review see Charnley, 2003) and **have been attributed** [present perfect] with insecticidal activity (Sree et al., 2008). Not all isolates of *Metarhizium* spp. **produce** [present] dtxs and consequently two strategies of fungal pathogenicity **have been proposed** [present perfect] (Kershaw et al., 1999). Some isolates **kill** [present] their host by proliferating in the haemocoel without producing toxins (growth strategy). Others **show** [present] limited growth prior to death and **employ** [present] dtxs to help overcome their host (toxin strategy). In reality there may **be** [present] a continuum between these two extreme positions (Charnley, 2003). Other secondary metabolites produced by *Metarhizium* spp. also **have** [present] likely roles in pathogenicity (Molnar et al., 2010). At least 38 dtxs or dtx analogues **have been isolated** [present perfect] to date and these **can be categorized** [present] in to 5 groups (A–E) based on chemical structure. Dtxs A, B and E, **are secreted** [present] during mycosis and **have been associated** [present perfect] with insecticidal activity; however, their exact role in pathogenesis **is** [present] not well understood (Amiri-Besheli et al., 2000; Kershaw et al., 1999; Samuels et al., 1988; Sree et al., 2008). A role in immunosuppression **is** [present] consistent with the evidence that dtx A **interferes** [present] with plasmatocyte attachment and spreading (Vilcinskas et al., 1997), nodulation (Huxham et al., 1989) and induction of humoral defence (Pal et al., 2007). In this study we **compared** [past] behavioural fever in locusts infected with two different isolates of *Metarhizium* spp.: *M. acridum* (IMI33018) which **employs** [present] the ‘‘growth strategy’’ and *Metarhizium robertsii* (ARSEF 2575) which **employs** [present] the ‘‘toxin strategy’’. IMI330189 in common with other members of *M. acridum* **does not produce** [present] dtxs (Kershaw et al., 1999; Freimoser et al., 2003). ARSEF 2575 **is** [present] a prolific producer of dtxs (Kershaw et al., 1999; Samuels et al., 1988). However, both fungal isolates **have** [present] similar temperature growth curves, with an optimum around 28–30 °C (Ouedraogo et al., 1997; Rangel et al., 2010 and data unpublished). We **hypothesized** [past] that since behavioural fever **is** [present] a component of the immune response, and dtx A **is** [present] known to interfere with immune defence, then presence or absence of dtx A during infection may **influence** [present] the extent and timing of fever.

2. Materials and methods

[Note the use of the passive voice throughout the Materials and methods section]

2.1. Maintenance of *S. gregaria*

Desert locusts, *S. gregaria* (Forskål) L. (Orthoptera: Acrididae) **were reared** [past] on a 12 h light:12 h dark photocycle in a controlled temperature room at 28 °C, 40% relative humidity. Each cage **was equipped** [past] with a 60W light bulb, providing a range of ambient temperatures. Locusts **were provided** [past] with wheat bran, distilled water, and fresh wheat shoots. Water **was** periodically **treated** [past] with a 5% antiprotozoal solution (w/v, 4.26% sodium sulfamethazine, 3.65% sodium sulfathiazole, 3.13% sodium sulfamerazine) to suppress growth of the sporozoan parasite, *Malamoeba* locusta (Tobe and Pratt, 1975). Male adult desert locusts, aged between 10–14 days **were used** [past] in all experiments.

2.2. Maintenance of *Metarhizium* spp.

Both strains of *Metarhizum* spp. used, *M. acridum* IMI330189 and *M. robertsii* ARSEF2575 (previously known as *Metarhizium anisopliae* var *acridum*, and *M. anisopliae* ME1, respectively (Bischoff et al., 2009), **were maintained** [past] at 28 °C in continuous light on ¼ strength Sabouraud’s dextrose agar (SDA) for 7–14 days.

2.3. Preparation of conidial spore suspensions

For inoculations, conidia **were suspended** [past] in cottonseed oil (Sigma-Aldrich). Ten millilitres of oil **was poured** [past] onto a sporulating plate and the conidia gently **dislodged** [past] using a sterile loop or spreader. To remove mycelia and large clumps of conidia, this suspension **was vortexed** [past] briefly, **passed** [past] through 4 layers of sterile muslin and then **placed** [past] in a sonicating water bath (15 °C for 5 min). Spore concentration **was determined** [past] using a Neubauer haemocytometer and **adjusted** [past] to 3.75×10^7 per ml. Only spore suspensions with greater than 95% germination rates **were used** [past] for experiments.

2.4. Treatment of *S. gregaria*

2.4.1. Inoculation with fungus

Prior to inoculation locusts **were chilled** [past] for 15 minutes at 4 °C. Locust **were** topically **inoculated** [past] with 2 µl of fungal suspension (equates to ca. 75,000 spores) under the pronotal shield using a hand microapplicator fitted with a 1 ml all glass syringe (Burkard Co.) and a sterile 15 gauge needle. Controls **were treated** [past] with cottonseed oil alone.

2.4.2. Injection of destruxin A

Locusts **were not chilled** [past] to avoid any influence this may have on temperature preference, but **were held** [past] at room temperature for 15 min prior to injection. Destruxin A (Sigma-Aldrich and a gift from Prof S E Reynolds, University of Bath) **was dissolved** [past] in Hoyle's saline (50 µg/10 µl) and **injected** [past] at a rate of 10 µl per gram of locust. Injections **were carried out** [past] using a hand microapplicator fitted with a 1 ml all glass syringe (Burkard Co.) and a 15 gauge needle which **was introduced** [past] dorsoventrally, breaking the intersegmental membrane between the 3rd and 4th abdominal segments. Immediately following injection, the abdomen **was** gently **pumped** [past] to promote distribution of the injected fluid. Controls **were treated** [past] with Hoyle's saline alone.

2.5. Recording mortalities and surface sterilisation treatment of cadavers

Cages **were checked** [past] daily for mortalities. Cadavers **were** surface **sterilized** [past] by sequential immersing in 1% bleach, sterile distilled water, 70% ethanol and sterile distilled water for ca. 20–30 s. Cadavers **were** then **placed** [past] in Petri dishes containing 2 sheets of Whatman No. 1 filter paper saturated with sterile distilled water to provide humidity. These **were kept** [past] at 28 °C under constant light, i.e. the optimum growth conditions for *Metarhizum* spp. for up to 14 days and the presence of fungal growth/sporulation on the cadaver surface **was recorded** [past].

2.6. Experimental set-up for recording the temperature of locusts

An aluminium cage **was designed** and **constructed** [past], specifically to incorporate an Indigo systems omega LVDS/RS-422 Infrared camera and to provide maximum image coverage of an experimental arena. The cage **consisted** [past] of an experimental arena (210 mm long × 250 mm high × 300 mm wide) attached to a funnel. The IR camera **was placed** [past] at the end of the funnel with a view to the main arena. A 60W light bulb set on a 12:12 h on-off cycle **was placed** [past] at the top of the cage above a mesh lid, creating a vertical thermal gradient over a climbing frame spanning the interior of the experimental arena. This **provided** [past] a temperature range ca. 28–55 °C during the photophase. During the scotophase no thermal gradient **was provided** [past] and an ambient temperature of 28 °C **was reached** [past]. Cohorts of 5 locusts from the same treatment group **were placed** [past] in the cage for each repeat. At the beginning of each repeat, enough food and water **was provisioned** [past] to last the entirety of the experiment, thereby minimising disturbance to the locusts. Prior to experiments, the IR camera **was calibrated** [past] against an adult male locust cadaver, aged 10–14 days, i.e. the same age and sex as locusts **used** [past] for experiments. For calibrations, IR measurements **were recorded** [past] simultaneously over a temperature range of 25–55 °C, with a K-type thermocouple **placed** [past] inside the thorax area of the cadaver. An Omega software programme **was adapted** [past] to capture data frames at regularly intervals from as little as 1 s apart with an optional start time delay. Raw data files **were viewed** [past] in MATLAB R2007a as false colour images on a 164 × 128 pixel matrix. The pixel area covering the thorax of individual

locusts **was highlighted** [past] and the median value of the highlighted pixels **used** [past] for temperature analysis. Following the method of Baughn et al. (1999), the data from each section **was processed** [past] using a 5 × 5 median filter (Medfilt2 in MATLAB).

2.7. Statistical analysis

Statistical analysis **was carried out** [past] with SPSS version 13.0 for Windows. Temperature preferences **were analysed** [past] with Linear Mixed Model (LMM) over multiple time points and ANOVA where individual time points **were tested** [past]. Survival analyses **were conducted** [past] using Kaplan–Meier and Cox-regression. Pathogen treatment and injection treatment **were set** [past] as categorical covariates.

3. Results

3.1. Behavioural fever response during mycosis with *Metarhizium* spp.

The body temperature for locusts inoculated with *M. acridum*, *M. robertsii* or cottonseed oil controls **were recorded** [past] at 24, 48, 72, 96 and 120 h post-inoculation (HPI). No mortalities **occurred** [past] during this time period. Control locusts **preferred** [past] 38.5 ± 0.54 °C and their temperature preference **did not change** [past] over time (Linear Mixed Model (LMM), $F = 0.326$, $p = 0.859$). Temperature preferences for *Metarhizium*-inoculated locusts **were** [past] similar to controls at 24 HPI (ANOVA, $F = 0.077$, $p = 0.926$). Fever responses **differed** [past] between the pathogen treatments. Locusts infected with *M. acridum* **displayed** [past] a prolonged fever, **observed** [past] from 48 HPI onwards with temperatures of 43.0 ± 0.69 °C. In comparison, only a shortlived fever of 42.2 ± 1.43 °C **was observed** [past] at 48 HPI for locusts infected with *M. robertsii*. Mean temperatures steadily **declined** [past] at subsequent time points to temperatures similar to those preferred by controls and fever **was not observed** [past] at any other time point (Fig. 1).

3.2. Effect of temperature on the mortality of *Metarhizium*-infected locusts

Preventing locusts from thermoregulating severely **reduced** [past] survival during mycosis. No difference, however, **was found** [past] between controls either maintained at a constant 28 °C or allowed to thermoregulate (Log Rank (Mantel-Cox) Expt. 1: $v2 = 0.601$, $p = 0.438$; Expt. 2: $v2 = 0.222$, $p = 0.64$). Locusts infected with *M. acridum* and provided with a thermal gradient **had** [past] an estimated median survival greater than 20 days (i.e. greater than the experimental duration observed here), significantly lower than controls allowed to thermoregulate (Log Rank (Mantel-Cox): $v2 = 4.27$, $p = 0.039$). In contrast, all infected locusts kept at 28 °C **were dead** [past] by 10 days PI and **had** [past] an estimated median survival time of 8 days, significantly lower than both controls and infected locusts allowed to thermoregulate (Control no thermal gradient: $v2 = 51.12$, $p < 0.0005$; Control thermal gradient: $v2 = 62.38$, $p < 0.0005$; Infected thermal gradient: $v2 = 51.12$, $p < 0.0005$). Temperature (Cox regression: Wald (W) = 24.46, Hazard ratio (HR) = 54.3, $p < 0.0005$) and a pathogen × temperature effect (W= 4.40, HR = 0.046, $p = 0.036$), but not pathogen treatment alone (W = 3.12, HR = 0.144, $p = 0.077$) significantly **contributed** [past] to the observed differences (Fig. 2a). Locusts infected with *M. robertsii* also **survived** [past] longer when allowed to thermoregulate ($v2 = 11.57$, $p = 0.001$). However, the effect **was** [past] not as prominent as that observed in locusts infected with *M. acridum*, and 50% of infected locusts kept at 28 °C **were** [past] still alive at day 10 PI. Estimated median survival time for infected locusts kept at 28 °C **was** [past] 6 days, significantly shorter than infected locusts allowed to thermoregulate. Temperature (W = 8.99, HR = 3.417, $p = 0.003$) and pathogen (W= 4.68, HR = 0.184, $p = 0.030$), but not a temperature × pathogen interaction (W= 0.62, HR = 0.455, $p = 0.431$) significantly **contributed** [past] to the observed differences (Fig. 2b). Cadavers **were** surface **sterilized** and **maintained** [past] at 28 °C under constant light and high humidity. Emergence of the fungus **was observed** [past] for >90% of cadavers previously inoculated with either *M. acridum* or *M. robertsii*, consistent with *Metarhizium* being the causative agent of death. No fungal growth **was observed** [past] on cadavers from control treatments.

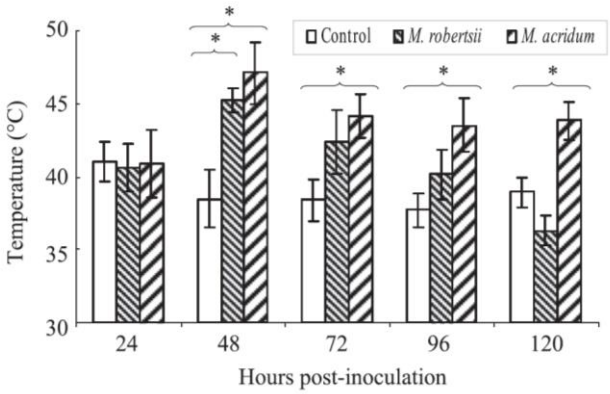

Fig. 1. Mean ± SE temperature preferences for locusts after inoculation with *Metarhizium robertsii*, *Metarhizium acridum* or cottonseed oil (controls). N = 10–15 per treatment. *p < 0.05.

3.3. Temperature preferences for *M. acridum*-infected locusts after injection with destruxin A

Locusts inoculated with *M. acridum* (i.e. induces a fever, but does not produce dtxs) **were injected** [past] with dtx A at either 22, 46 or 70 HPI, and their temperature preferences **recorded** [past]. Destruxin A **had** [past] an inhibitory effect on behavioural fever; however, this **was** [past] variable depending on the timing of dtx A administration. Inhibition **was** [past] most effective when injected at an earlier stage in mycosis. Temperature preferences for locusts from all treatments **were** [past] similar to controls at 24 HPI (22 HPI: t = −0.174, p = 0.863; 46 HPI t = −1.002, p = 0.326; 70 HPI t = 0.121, p = 0.905). Dtx A injection at 22 HPI **was** [past] sufficient to inhibit fever altogether and temperature preferences **were** [past] similar to controls at all time points (48 HPI, t = 2.759, p = 0.012; 72 HPI, t = 3.130, p = 0.006; 96 HPI, t = 2.373, p = 0.030). Injection at 46 HPI **inhibited** [past] fever at 72 HPI, but not at later time points (48 HPI, t = −0.151, 0.882; 72 HPI, t = 2.849, p = 0.012; 96 HPI, t = 1.811, p = 0.09). Injection at 70 HPI **had** [past] no inhibitory effect (48 HPI, t = −0.059, p = 0.214; 72 HPI, t = 0.986, p = 0.339; 96 HPI, t = 0.986, p = 0.339) (Fig. 3). Injection of dtx A alone **had** [past] no effect on temperature preferences, which **were** [past] similar to those of controls, with overall mean ± SE of 37.3 ± 0.4 d and 37.5 ± 0.3 d, for Oil + Saline and Oil + dtx A, respectively (ANOVA: 22HPI treatment, F = 0.244, p = 0.973; 46 HPI treatment, F = 1.497, p = 0.187; 70 HPI treatment, F = 0.249, p = 0.971).

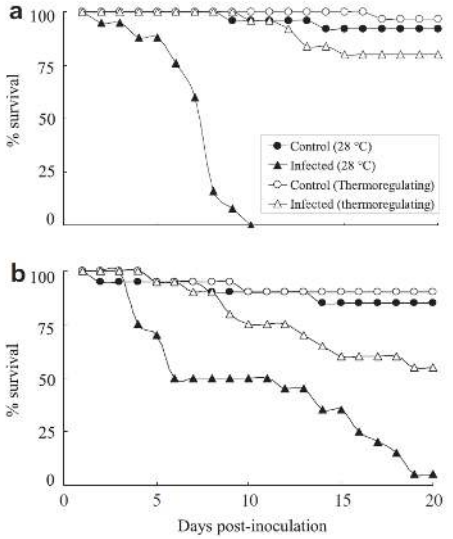

Fig. 2. Survival curves of locusts inoculated with (a) *Metarhizium acridum* and (b) *Metarhizium robertsii*, and either allowed to thermoregulate on a temperature gradient or maintained at a constant 28 °C. Controls **are treated** [present] with cottonseed oil only. N = 20–30 per treatment.

3.4. Effect of destruxin A on survival of mycosed locusts

Injection of dtx A **reduced** [past] the median survival time of locusts already infected with *M. acridum*. The effect **was** [past] greatest when dtx A was injected at 22 HPI, where median survival time was 10 d, at least 67% lower than locusts infected with *M. acridum* alone (Log Rank (Mantel-Cox): $X^2 = 31.18$, $p < 0.0005$). Locusts injected at 46 and 70 HPI **had** [past] median survival times of 14 and >30 d, respectively. However, a reduction in survival **was** [past] only significant at 46 HPI ({2 = 14.62, p = 0.002; 70 HPI: $X^2 = 4.48$, p = 0.214). For locusts receiving a second treatment at 22 HPI, both the initial pathogen treatment (Cox regression: W= 9.02, HR = 0.098, p = 0.003) and injection treatment effects (W= 5.66, HR = 2.508, p = 0.017) significantly **contributed** [past] to the observed differences. For treatments at 46 HPI, only initial pathogen treatment **was** [past] a significant variable (W= 7.62, HR = 0.205, p = 0.006) (Fig. 4).

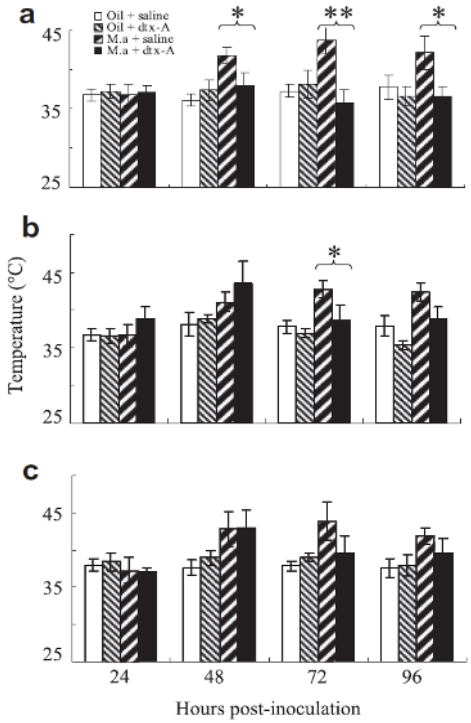

Fig. 3. Mean ± SE temperature preferences for locusts inoculated with *Metarhizium acridum* or cottonseed oil at time 0, followed by injection with destruxin A or Hoyle's saline at either (a) 22 (b) 46 (c) 70 h post-inoculation. N = 10–15 per treatment. *p < 0.05, **p < 0.01.

4. Discussion

During an infection the desert locust behaviourally **fevers** [present] by seeking out higher resting temperatures. In response to *M. acridum* infection, a prolonged fever **occurred** [past] from 48 to 120 HPI. This **is** [present] consistent with previous studies using *M. acridum* and other entomopathogens such as *Serratia marcescens* (Blanford and Thomas, 1999; Bundey et al., 2003). In contrast, only a short-lived fever **was observed** [past] at 48 HPI during mycosis with *M. robertsii*. Analogous to the physiological fever experienced in mammals, behavioural fever directly **impacts** [present] on pathogen growth and **can improve** [present] efficiency of the host immune system, thus extending the lifespan of the host (Arthurs and Thomas, 2001; Blanford and Thomas, 2001; Kluger, 1986; Ouedraogo et al., 2002).

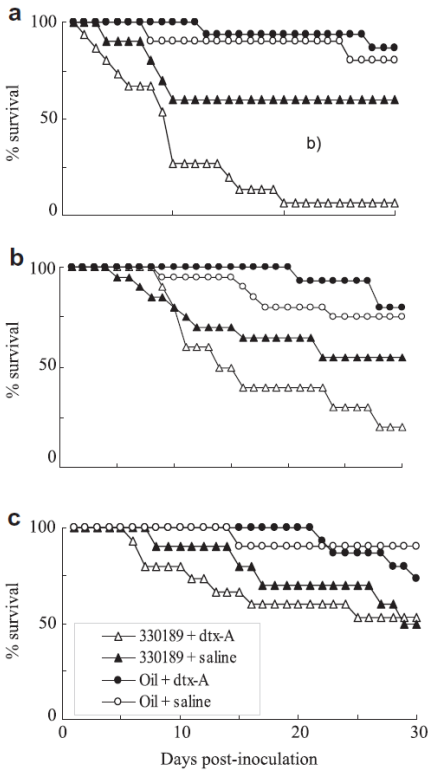

Fig. 4. Survival curves of locusts inoculated with *Metarhizium acridum* or cottonseed oil at time 0, followed by injection with destruxin A or Hoyle's saline at either (a) 22 (b) 46 (c) 70 h post inoculation. N = 10–20 per treatment.

For both fungal isolates, survival **was reduced** [past] when locusts were prevented from fevering during mycosis. Survival of locusts infected with *M. acridum* and allowed to thermoregulate freely **was** [past] comparable to similar studies using 5th instar and adult *S. gregaria* (Blanford and Thomas, 1999; Elliot et al., 2002). Blanford and Klass (2004) **have** previously **assessed** [present perfect] survival of locusts in the field under varied thermal environmental conditions. The reduced survival of *M. acridum*-infected locusts restricted from fevering found here **was** [past] similar to locusts exposed to thermal field conditions favourable to the fungus (i.e. daytime <38 °C, night-time >20 °C) (Blanford and Klass, 2004). This **is** [present] not surprising given that restriction of fever resulted in host and pathogen spending prolonged periods of time close to the optimal fungal growth temperature. At 28 °C *M. acridum* **was** [past] more virulent than *M. robertsii*. However, when locusts were allowed to thermoregulate *M. robertsii* **was** [past] more virulent, and *M. acridum*-infected locusts **had** [past] survival patterns more similar to controls. Both isolates **have** [present] similar thermal growth curves and **cease** [present] to grow above 40 °C (Ouedraogo et al., 1997; Rangel et al., 2010). This, at least in part, **is** [present] likely to represent the different thermal regimes associated with each isolate. Survival benefits **are** [present] only maintained for the duration of the fever response (Ouedraogo et al., 2004). The short-lived fever of locusts infected with *M. robertsii* **is** [present] therefore likely to offer little advantage compared to that in locusts infected with *M. acridum*, where fever **was expressed** [past] throughout the photophase during the period of observation. This indeed **appeared** [past] to be the case when survival curves of the two cohorts of locusts **were compared** [past].

Administration of dtx A during infection with *M. acridum* **inhibited** [past] behavioural fever. This **was** [past] most effective at 22 HPI, around the time at which the fungus **penetrates** [present] the cuticle and **enters** [present] the haemolymph (Gunnarsson, 1988). At this point, early stages of pathogen-recognition **occur** [present] in the haemolymph and suppression of the immune system may **provide** [present] the fungus with a greater survival advantage. This would **enable** [present] *M. acridum* to allocate resources to growth, rather than combating host defence. An inhibitory role of

dtxs on the insect immune system **is** [present] further supported by evidence of interference with plasmatocytes involved in encapsulation and phagocytosis (Vilcinskas et al., 1997), inhibition of nodulation (Huxham et al., 1989) and a down regulation of antimicrobial peptides (Pal et al., 2007). Suppression of such diverse immune defences **infers** [present] dtxs target early components of the pathogen recognition or immune response pathways. Inhibition of behavioural fever reported here may likewise **be targeted** [present] by dtxs as a component of the immune response. Destruxins **are** [present] not the only secondary metabolites produced by *Metarhizium* spp. and attributed with a role in pathogenicity (Molnar et al., 2010). Toxins which **are not** well **characterized** [present] or **are** [present] as yet unknown may also **play** [present] a role in suppression of the insect immune system including behavioural fever. Studies identifying genes such as those found for *M. acridum* mycosis of locust wings (He and Xia, 2009) and genome sequencing of *Metarhizium* isolates **will help** [future] elucidate the array of secondary metabolites involved in pathogenicity (Gao et al., 2011; Molnar et al., 2010).

Locusts injected with dtx A **were** [past] more susceptible to infection with an isolate of *Metarhizium* spp. that does not itself produce this toxin. It is unclear whether the reduction in survival **was** [past] due to a lack of fever per se, or whether additionally the inhibition of other immune defences by dtx or other unknown factors **played** [past] a part. The isolate in question, *M. acridum* IMI330189, **is** [present] the active constituent of one of the biopesticides presently used in Africa for locust control (Lomer et al., 2001). Since the fever response **is thought** [present] to play a major part in increasing time to kill during field applications, similarly engineering IMI330189 to synthesise dtx A, may **improve** [present] biopesticide efficiency. Dtxs **are thought to be synthesised** [present] non-ribosomally by a thiotemplate mechanism (Jegorov et al., 1993). To date the destruxin synthetase **has not been identified** [present perfect], but comparable enzymes in other fungi suggests it **is** [present] likely to be a very large protein, e.g. in the order of 350–1600 kDa and the product of an equally large gene. Thus the molecular biology would **be** technically **challenging** [present] and such an enzyme **is** [present] likely to synthesize a number of secondary metabolites in addition to dtx A (Marahiel et al., 1997). However, an advantage of this approach **is** [present] that the target gene comes from a related organism. Furthermore, destruxin-producing *Metarhizium* spp. **are** [present] naturally found in soil and **are** already **registered** [present] for use as biopesticides. Cause for environmental concern **is** therefore **reduced** [present] in comparison to isolates of *Metarhizium* spp. genetically engineered to express neurotoxins from scorpions (Lu et al., 2008). Under field conditions, virulence of *M. acridum* **can vary** [present] considerably over spatial scales and this **is** [present] highly dependent on thermal conditions. Targeting applications to areas or times where locusts are most vulnerable to infection **will** further **aid** [future] efficiency (Klass et al., 2007). Environmental modelling of an engineered biopesticide, similar to that carried out for wild-type *M. acridum* by Klass et al. (2007), **would be** [present] indicative of the most effective field conditions for application.

The amounts of dtx injected in the present work **are** [present] likely to be greater than those determined in haemolymph during mycosis. There **are** [present] no figures available for the isolate and insect used here. However, no direct comparison could **be made** [present] since hyphal bodies in the haemocoel **are** [present] likely to provide locally high doses of dtx around aggregating haemocytes which could not **be equated** [present] with overall haemolymph concentrations. The impact of sephadex beads coated with dtx beds on phagocytic haemocytes **illustrates** [present] this principle well (Huxham et al., 1989). Furthermore, metabolic detoxification of dtxs by insect hosts of dtx-producing fungi means it **is** [present] difficult to quantify dtx levels *in vivo* (Soledade et al., 2002).

Bundey et al. (2003) **have shown** [present perfect] previously that, in common with the fever response in mammals, eicosanoids **play** [present] a part in the regulation of behavioural fever in locusts (Bundey et al., 2003). Fever **is** [present] an element of the innate immune response which itself **is** [present] conserved across the phyla (Blatteis, 2003). Thus the use of dtx as a tool to investigate regulation of fever may **have** [present] value beyond the confines of entomology.