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

1. Introduction

Isolates of the fungal entomopathogen Metarhizium spp. have been developed as biopesticides against a range of insects as an alternative to chemical pesticides. A formulation of the entomopathogen Metarhizium acridum (IMI330189) has been developed 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 by seeking out higher environmental temperatures than their healthy conspecifics (Bundey et al., 2003; Elliot et al., 2002). The temperatures achieved are suboptimal for pathogen growth (Arthurs and Thomas, 2001; Blanford and Thomas, 2001) and enhance other aspects of the immune response n.b. behavioural fever is itself a component of immune defence (Ouedraogo et al., 2002). The survival advantage provided by behavioural fever is 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 to identify ways of interfering with the fever response. Behavioural fever has been reported 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 (McClein et al., 1988) and Lepidoptera (Karban, 1998), though it has been best characterized 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 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 largely unknown. Evidence from injecting locusts with inhibitory chemicals of the same pathways does however indicate similar mechanisms have been conserved (Bundey et al., 2003). Toxic secondary metabolites, of which the
Destruxins (dtxs), a family of cyclic depsipeptides, are the most abundant. They have been identified from a number of Metarhizium spp. isolates. These toxins have a wide variety of effects in vitro (for review see Charnley, 2003) and have been attributed with insecticidal activity (Sree et al., 2008). Not all isolates of Metarhizium spp. produce dtxs and consequently two strategies of fungal pathogenicity have been proposed (Kershaw et al., 1999). Some isolates kill their host by proliferating in the haemocoel without producing toxins (growth strategy). Others show limited growth prior to death and employ dtxs to help overcome their host (toxin strategy). In reality there may be a continuum between these two extreme positions (Charnley, 2003). Other secondary metabolites produced by Metarhizium spp. also likely roles in pathogenicity (Molnar et al., 2010). At least 38 dtxs or dtx analogues have been isolated to date and these can be categorized in to 5 groups (A–E) based on chemical structure. Dtxs A, B and E are secreted during mycosis and have been associated with insecticidal activity; however, their exact role in pathogenesis is 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 consistent with the evidence that dtx A interferes 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 behavioural fever in locusts infected with two different isolates of Metarhizium spp.: M. acridum (IMI33018) which employs the “growth strategy” and Metarhizium robertsii (ARSEF 2575) which employs the “toxin strategy”. IMI330189 in common with other members of M. acridum does not produce dtxs (Kershaw et al., 1999; Freimoser et al., 2003). ARSEF 2575 is a prolific producer of dtxs (Kershaw et al., 1999; Samuels et al., 1988). However, both fungal isolates have similar temperature growth curves, with an optimum around 28–30 °C (Ouedraogo et al., 1997; Rangel et al., 2010 and data unpublished). We hypothesized that since behavioural fever is a component of the immune response, and dtx A is known to interfere with immune defence, then presence or absence of dtx A during infection may influence 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 (Forschål) L. (Orthoptera: Acrididae) were reared on a 12 h light:12 h dark photocycle in a controlled temperature room at 28 °C, 40% relative humidity. Each cage was equipped with a 60W light bulb, providing a range of ambient temperatures. Locusts were provided with wheat bran, distilled water, and fresh wheat shoots. Water was periodically treated 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 in all experiments.

2.2. Maintenance of Metarhizium spp.

Both strains of Metarhizium 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 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 in cottonseed oil (Sigma-Aldrich). Ten millilitres of oil was poured onto a sporulating plate and the conidia gently dislodged using a sterile loop or spreader. To remove mycelia and large clumps of conidia, this suspension was vortexed briefly, passed through 4 layers of sterile muslin and then placed in a sonicating water bath (15 °C for 5 min). Spore concentration was determined using a Neubauer haemocytometer and adjusted to 3.75 × 10⁷ per ml. Only spore suspensions with greater than 95% germination rates were used for experiments.

2.4. Treatment of S. gregaria

2.4.1. Inoculation with fungus

Prior to inoculation locusts were chilled for 15 minutes at 4 °C. Locusts were topically inoculated 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 with cottonseed oil alone.

2.4.2. Injection of destruxin A

Locusts were not chilled to avoid any influence this may have on temperature preference, but were held 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 in Hoyle’s saline (50 µg/10 µl) and injected at a rate of 10 µl per gram of locust. Injections were carried out using a hand microapplicator fitted with a 1 ml all glass syringe (Burkard Co.) and a 15 gauge needle which was introduced dorsoventrally, breaking the intersegmental membrane between the 3rd and 4th abdominal segments. Immediately following injection, the abdomen was gently pumped to promote distribution of the injected fluid. Controls were treated with Hoyle’s saline alone.

2.5. Recording mortalities and surface sterilisation treatment of cadavers

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

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

An aluminium cage was designed and constructed, 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 of an experimental arena (210 mm long × 250 mm high × 300 mm wide) attached to a funnel. The IR camera was placed 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 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 a temperature range ca. 28–55 °C during the photophase. During the scotophase no thermal gradient was provided and an ambient temperature of 28 °C was reached. Cohorts of 5 locusts from the same treatment group were placed in the cage for each repeat. At the beginning of each repeat, enough food and water was provisioned to last the entirety of the experiment, thereby minimising disturbance to the locusts. Prior to experiments, the IR camera was calibrated against an adult male locust cadaver, aged 10–14 days, i.e. the same age and sex as locusts used for experiments. For calibrations, IR measurements were recorded simultaneously over a temperature range of 25–55 °C, with a K-type thermocouple placed inside the thorax area of the cadaver. An Omega software programme was adapted 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 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 $5 \times 5$ 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


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 on wards 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: $v^2 = 0.601$, $p = 0.438$; Expt. 2: $v^2 = 0.222$, $p = 0.64$). 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): $v^2 = 4.27$, $p = 0.039$). 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: $v^2 = 51.12$, $p < 0.0005$; Control thermal gradient: $v^2 = 62.38$, $p < 0.0005$; Infected thermal gradient: $v^2 = 51.12$, $p < 0.0005$). 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, $p = 0.036$) significantly contributed [past] to the observed differences (Fig. 2a). Locusts infected with *M. robertsii* also survived [past] longer when allowed to thermoregulate ($v^2 = 11.57$, $p = 0.001$). 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 dead [past] 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.
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 with dtx A at either 22, 46 or 70 HPI, and their temperature preferences were recorded. Destruxin A had an inhibitory effect on behavioural fever; however, this was variable depending on the timing of dtx A administration. Inhibition was most effective when injected at an earlier stage in mycosis. Temperature preferences for locusts from all treatments were 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 sufficient to inhibit fever altogether and temperature preferences were 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 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 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 no effect on temperature preferences, which were 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, $F = 0.249, p = 0.971$).
3.4. Effect of destruxin A on survival of mycosed locusts

Injection of dtx A reduced the median survival time of locusts already infected with *M. acridum*. The effect was 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 median survival times of 14 and >30 d, respectively. However, a reduction in survival was only significant at 46 HPI ($X^2 = 14.62$, $p = 0.002$; 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 to the observed differences. For treatments at 46 HPI, only initial pathogen treatment was 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 by seeking out higher resting temperatures. In response to *M. acridum* infection, a prolonged fever occurred from 48 to 120 HPI. This is 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 at 48 HPI during mycosis with *M. robertsii*. Analogous to the physiological fever experienced in mammals, behavioural fever directly impacts on pathogen growth and can improve 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).
For both fungal isolates, survival was reduced when locusts were prevented from fevering during mycosis. Survival of locusts infected with *M. acridum* and allowed to thermoregulate freely was 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 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 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 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 more virulent than *M. robertsii*. However, when locusts were allowed to thermoregulate *M. robertsii* was more virulent, and *M. acridum*-infected locusts had survival patterns more similar to controls. Both isolates have similar thermal growth curves and cease to grow above 40 °C (Ouedraogo et al., 1997; Rangel et al., 2010). This, at least in part, is likely to represent the different thermal regimes associated with each isolate.

Survival benefits are only maintained for the duration of the fever response (Ouedraogo et al., 2004). The short-lived fever of locusts infected with *M. robertsii* therefore likely to offer little advantage compared to that in locusts infected with *M. acridum*, where fever was expressed throughout the photophase during the period of observation. This appeared to be the case when survival curves of the two cohorts of locusts were compared.

Administration of dtx A during infection with *M. acridum* inhibited behavioural fever. This was most effective at 22 HPI around the time at which the fungus penetrates the cuticle and enters the haemolymph (Gunnarsson, 1988). At this point, early stages of pathogen-recognition occur in the haemolymph and suppression of the immune system may provide the fungus with a greater survival advantage. This would enable *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 synthesise 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 haemocoeel 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.