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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 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 short-lived 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 (McClain et al., 1988) and Lepidoptera (Karban, 1998), though it has been best characterised 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, 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 have likely roles in pathogenicity (Molnar et al., 2010).

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At least 38 dtxs or dtx analogues have been isolated to date and these can be categorised 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 hypothesised 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 method

2.1. Maintenance of S. gregaria

Desert locusts, *S. gregaria* (Forskål) 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 60 W 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 *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 at 28 °C in continuous light on 1/4 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 3.75×10^7 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. Locust were topically inoculated with 2μl 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 sterilised 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 *Metarhizum* 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 60 W 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