

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** [subject – plural] behaviourally **fever** [verb – plural] by seeking out higher environmental temperatures. **This behaviour** [subject – singular] **places** [verb – singular] the pathogen at sub-optimal growth temperatures while improving the efficiency of the immune system, thereby prolonging the lifespan of the host. **It** [subject – singular] **is** [verb – singular] 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** [subject – plural] **investigated** [simple past] the behavioural fever response of desert locusts to two fungal pathogens. **A** prolonged **fever** [subject – singular] **was** [verb – singular] observed in locusts infected with *Metarhizium acridum*. However, **fever** [subject – singular] **was** [verb – singular] comparatively shortlived during infection with *Metarhizium robertsii*. In both cases, **restriction of thermoregulation*** [subject – singular] **reduced** [simple past] lifespan.

*The true subject of a sentence often comes before the word “of”.
e.g., “The **toxicity** [subject – singular] **of** three compounds **was** [verb – singular] measured ...”

Destruxin A (dtx A) [subject – singular] produced by *M. robertsii*, but not *M. acridum**, **has** [verb – singular] previously been associated with the inhibition of the insect immune system.

*Asides don’t make a subject plural e.g., **This result** [subject – singular], *as well as those from other studies* [does not influence subject-verb agreement], **supports** [verb – singular] our hypothesis

Injection [subject – singular] of dtx A during infection with the fever causing *M. acridum* **inhibited** [simple past] fever and **was** [verb – singular] particularly effective when administered early on in infection. Furthermore, **locusts** [subject – plural] injected with dtx A **were** [verb – plural] more susceptible to *M. acridum* infection. Therefore, **engineering *M. acridum* isolates currently used for locust biocontrol to express dtx A*** [a clause serving as the subject] **may** [helping verb] improve efficiency of control by interfering with fever.

* A phrase or clause serving as the subject takes a singular verb.
e.g., **Interpreting these results** [clause serving as the subject] **was** [verb – singular] difficult at first.
Interpreting this result [clause serving as the subject] **was** [verb – singular] difficult at first.

Of course, these rules do not apply when using helping verbs, such as *can, could, should, may, might, will, would, must*.

1. Introduction

Isolates [subject – plural] of the fungal entomopathogen *Metarhizium* spp. **have** [verb – plural] been developed as biopesticides against a range of insects as an alternative to chemical pesticides. **A formulation** [subject – singular] of the entomopathogen *Metarhizium acridum* (IMI330189) **has** [verb

– **singular**] 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** [subject – **plural**] behaviourally **fever** [verb – **plural**] by seeking out higher environmental temperatures than their healthy conspecifics (Bundey et al., 2003; Elliot et al., 2002). The **temperatures** [subject – **plural**] achieved **are** [verb – **plural**] suboptimal for pathogen growth (Arthurs and Thomas, 2001; Blanford and Thomas, 2001) and **enhance** [verb – **plural**] other aspects of the immune response n.b. behavioural **fever** [subject – **singular**] **is** [verb – **singular**] itself a component of immune defense (Ouedraogo et al., 2002). **The** survival **advantage** [subject – **singular**] provided by behavioural fever **is** [verb – **singular**] 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** [subject – **singular**] of improving the efficacy of fungal biocontrol **may** [helping verb] be to identify ways of interfering with the fever response.

Behavioural **fever** [subject – **singular**] **has** [verb – **singular**] 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** [subject – **singular**] **has** [verb – **singular**] 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** [subject – **singular**] **has** [verb – **singular**] 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** [subject – **plural**] involved in behavioural fever **are** [verb – **plural**] largely unknown. **Evidence** [subject – **singular**] from injecting locusts with inhibitory chemicals of the same pathways **does** [helping verb] however indicate similar **mechanisms** [subject – **plural**] **have** [verb – **plural**] been conserved (Bundey et al., 2003). Toxic secondary **metabolites** [subject – **plural**], of which the **destruxins (dtxs)** [subject – **plural**], a family of cyclic depsipeptides, **are** [verb – **plural**] the most abundant, **have** [verb – **plural**] been identified from a number of *Metarhizium* spp. isolates.*

*This sentence has two parts, each of which obeys the subject-verb agreement rules: 1: Toxic secondary **metabolites** [subject – **plural**] **have** [verb – **plural**] been identified from a number of *Metarhizium* spp. isolates. 2: of which the **destruxins (dtxs)** [subject – **plural**], a family of cyclic depsipeptides, **are** [verb – **plural**] the most abundant.

These toxins [subject – **plural**] **have** [verb – **plural**] a wide variety of effects *in vitro* (for review see Charnley, 2003) and **have** [verb – **plural**] been attributed with insecticidal activity (Sree et al., 2008). Not all **isolates** [subject – **plural**] of *Metarhizium* spp. **produce** [verb – **plural**] dtxs and consequently two **strategies** [subject – **plural**] of fungal pathogenicity **have** [verb – **plural**] been proposed (Kershaw et al., 1999). Some **isolates** [subject – **plural**] **kill** [verb – **plural**] their host by proliferating in the haemocoel without producing toxins (growth strategy). **Others** [subject – **plural**] **show** [verb – **plural**] limited growth prior to death and **employ** [verb – **plural**] dtxs to help overcome their host (toxin strategy). In reality, **there** [subject – **singular**] **may** [helping verb] be a continuum between these two extreme positions (Charnley, 2003). Other secondary **metabolites** [subject – **plural**] produced by *Metarhizium* spp. also **have** [verb – **plural**] likely roles in pathogenicity (Molnar et al., 2010). At least **38 dtxs or dtx analogues** [plural subject *or* plural subject] **have** [verb – **plural**] been isolated to date and **these** [subject – **plural**] **can** [helping verb] be categorized into 5 groups (A–E) based on chemical structure. Dtx A, B and E [**singular** subject, **singular** subject *and* **singular** subject] **are** [verb – **plural**] secreted during mycosis and **have** [verb – **plural**] been associated with insecticidal activity; however, their exact **role** [subject – **singular**] in pathogenesis **is** [verb – **singular**] not well understood (Amiri-Besheli et al., 2000; Kershaw et al., 1999; Samuels et al., 1988; Sree et al., 2008). **A role** [subject – **singular**] in immunosuppression **is** [verb – **singular**] consistent with the evidence that **dtx A** [subject – **singular**] **interferes** [verb – **singular**] with plasmatocyte attachment and spreading (Vilcinskis et al., 1997), nodulation (Huxham et al., 1989) and induction of humoral defence (Pal et al., 2007). In this study, **we** [subject – **plural**] **compared** [simple past] behavioural fever in locusts infected with two different isolates of *Metarhizium* spp.: ***M. acridum* (IMI33018)** [subject – **singular**]

which **employs** [verb – singular] the “growth strategy” and *Metarhizium robertsii* (ARSEF 2575) [subject – singular] which **employs** [verb – singular] the “toxin strategy”. **IMI330189** [subject – singular] in common with other members of *M. acridum* **does** [helping verb] not produce dtxs (Kershaw et al., 1999; Freimoser et al., 2003). **ARSEF 2575** [subject – singular] **is** [verb – singular] a prolific producer of dtxs (Kershaw et al., 1999; Samuels et al., 1988). However, both fungal **isolates** [subject – plural] **have** [verb – plural] similar temperature growth curves, with an optimum around 28–30 °C (Ouedraogo et al., 1997; Rangel et al., 2010 and data unpublished). **We** [subject – plural] **hypothesized** [simple past] that since behavioural **fever** [subject – singular] **is** [verb – singular] a component of the immune response, and **dtx A** [subject – singular] **is** [verb – singular] known to interfere with immune defence, then **presence or absence** [singular subject **or** singular subject] of dtx A during infection **may** [helping verb] influence the extent and timing of fever.

2. Materials and methods

2.1. Maintenance of *S. gregaria*

Desert **locusts** [subject – plural], *S. gregaria* (Forskål) L. (Orthoptera: Acrididae) **were** [verb – plural] reared on a 12 h light:12 h dark photoperiod in a controlled temperature room at 28°C, 40% relative humidity. Each **cage** [subject – singular] **was** [verb – singular] equipped with a 60W light bulb, providing a range of ambient temperatures. **Locusts** [subject – plural] **were** [verb – plural] provided with wheat bran, distilled water, and fresh wheat shoots. **Water** [subject – singular] **was** [verb – singular] 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** [subject – plural], aged between 10–14 days **were** [verb – plural] used in all experiments.

2.2. Maintenance of *Metarhizium* spp.

Both **strains** [subject – plural] 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** [verb – plural] 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** [subject – plural] **were** [verb – plural] suspended in cottonseed oil (Sigma-Aldrich). **Ten millilitres of oil*** [subject – singular] **was** [verb – singular] poured onto a sporulating plate and the **conidia** [subject – plural] gently **dislodged** [simple past] using a sterile loop or spreader.

*Subjects that are measurements/quantities (e.g., Ten millilitres, 5 g) are considered a single entity and so take a singular verb – i.e., “**5 g** [subject – singular] **was** [verb – singular] added to ...” = CORRECT
“**5 g** [subject – singular] **were** [verb – singular] added to ...” = INCORRECT

To remove mycelia and large clumps of conidia, **this suspension** [subject – singular] **was** [verb – singular] vortexed briefly, **passed** [simple past] through four layers of sterile muslin and then **placed** [simple past] in a sonicating water bath (15°C for 5 min). Spore **concentration** [subject – singular] **was** [verb – singular] determined using a Neubauer haemocytometer and **adjusted** [simple past] to 3.75×10^7 per ml. Only spore **suspensions** [subject – plural] with greater than 95% germination rates **were** [verb – plural] used for experiments.

2.4. Treatment of *S. gregaria*

2.4.1. Inoculation with fungus

Prior to inoculation, **locusts** [subject – plural] **were** [verb – plural] chilled for 15 minutes at 4°C. **Locusts** [subject – plural] **were** [verb – plural] 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** [subject – plural] **were** [verb – plural] treated with cottonseed oil alone.

2.4.2. Injection of destruxin A

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

2.5. Recording mortalities and surface sterilisation treatment of cadavers

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

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

An aluminium **cage** [subject – singular] **was** [verb – singular] 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** [subject – singular] **consisted** [simple past] of an experimental arena (210 mm long × 250 mm high × 300 mm wide) attached to a funnel. **The IR camera** [subject – singular] **was** [verb – singular] placed at the end of the funnel with a view to the main arena. **A** 60W light **bulb** [subject – singular] set on a 12:12 h on-off cycle **was** [verb – singular] 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** [subject – singular] **provided** [simple past] a temperature range ca. 28–55°C during the photophase. During the scotophase, no thermal **gradient** [subject – singular] **was** [verb – singular] provided and **an** ambient **temperature** [subject – singular] of 28 °C **was** [verb – singular] reached. **Cohorts** [subject – plural] of five locusts from the same treatment group **were** [verb – plural] placed in the cage for each repeat. At the beginning of each repeat, **enough food and water*** [enough + singular subject and singular subject] **was** [verb – singular] provisioned to last the entirety of the experiment, thereby minimising disturbance to the locusts.

*Indefinite quantifiers like “enough”, “all”, “any”, “more”, “most”, “none” and “some” take a singular verb when used with uncountable nouns (e.g., food and water) and a plural verb when used with countable nouns.

Prior to experiments, **the IR camera** [subject – singular] **was** [verb – singular] 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** [subject – plural] **were** [verb – plural] 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** [subject – singular] **was** [verb – singular] adapted to capture data frames at regularly intervals from as little as 1 s apart with an optional start time delay. Raw data **files** [subject – plural] **were** [verb – plural] viewed in MATLAB R2007a as false colour images on a 164 × 128 pixel matrix. **The pixel area** [subject – singular] covering the thorax of individual locusts **was** [verb – singular] highlighted and **the median value** [subject – singular] of the highlighted pixels **used** [simple past] for temperature analysis. Following the method of Baughn et al. (1999), **the data** [subject – plural] from each section **were** [verb – plural] processed using a 5 × 5 median filter (Medfilt2 in MATLAB).

2.7. Statistical analysis

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

3. Results

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

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

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

Preventing locusts from thermoregulating* [a clause serving as the subject] severely **reduced** [simple past] survival during mycosis. No **difference** [subject – singular], however, **was** [verb – singular] found 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** [subject – plural] infected with *M. acridum* and provided with a thermal gradient **had** [simple 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** [subject – plural] kept at 28 °C **were** [verb – plural] dead by 10 days PI and **had** [simple 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, $p = 0.036$), but not pathogen

treatment [singular subject *and* singular subject *but not* singular subject] alone (W = 3.12, HR = 0.144, p = 0.077) significantly **contributed** [simple past] to the observed differences (Fig. 2a). **Locusts** [subject – plural] infected with *M. robertsii* also **survived** [simple past] longer when allowed to thermoregulate (v2 = 11.57, p = 0.001). However, **the effect** [subject – singular] **was** [verb – singular] not as prominent as that observed in locusts infected with *M. acridum*, and **50% of infected locusts*** [subject – plural] kept at 28°C **were** [verb – plural] still alive at day 10 PI.

* Fractions and percentages (like indefinite quantifiers – see above) take a singular verb when used with uncountable nouns and a plural verb when used with countable nouns.
 e.g., “50% of **the** treatment **group** [subject – singular] **was** [verb – singular] ...”
 “50% of **the locusts** [subject – plural] **were** [verb – plural] ...”

Estimated median survival **time** [subject – singular] for infected locusts kept at 28 °C **was** [verb – singular] 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) [singular subject *and* singular subject *but not* singular subject] significantly **contributed** [simple past] to the observed differences (Fig. 2b). **Cadavers** [subject – plural] **were** [verb – plural] surface sterilized and maintained at 28°C under constant light and high humidity. **Emergence** [subject – singular] of the fungus **was** [verb – singular] observed 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** [subject – singular] **was** [verb – singular] observed 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 recorded*.

*This sentence has two parts, each of which obeys the subject-verb agreement rules:

- 1: **Locusts** [subject – plural] inoculated with *M. acridum* **were** [verb – plural] injected with dtx A at either 22, 46 or 70 HPI, and their temperature **preferences** [subject – plural] **recorded** [simple past];
- 2: ***M. acridum*** [subject of the parenthesis – singular] (i.e. **induces** [subject – singular] a fever, but **does** [helping verb] not produce dtxs)

Destruxin A [subject – singular] **had** [simple past] an inhibitory effect on behavioural fever; however, **this** [subject – singular] **was** [verb – singular] variable depending on the timing of dtx A administration. **Inhibition** [subject – singular] **was** [verb – singular] most effective when injected at an earlier stage in mycosis. Temperature **preferences** [subject – plural] for locusts from all treatments **were** [verb – plural] 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** [subject – singular] at 22 HPI **was** [subject – singular] sufficient to inhibit fever altogether and temperature **preferences** [subject – plural] **were** [verb – plural] 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** [subject – singular] at 46 HPI **inhibited** [simple 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** [subject – singular] at 70 HPI **had** [simple 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** [subject – singular] of dtx A alone **had** [simple past] no effect on temperature **preferences** [subject – plural], which **were** [verb – plural] 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 [subject – singular] of dtx A **reduced** [simple past] the median survival time of locusts already infected with *M. acridum*. **The effect** [subject – singular] **was** [verb – singular] greatest when dtx A was injected at 22 HPI, where median survival **time** [subject – singular] **was** [verb – singular] 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** [subject – plural] injected at 46 and 70 HPI **had** [simple past] median survival times of 14 and >30 d, respectively. However, **a reduction** [subject – singular] in survival **was** [verb – singular] only significant at 46 HPI ($\chi^2 = 14.62$, $p = 0.002$; 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** ($W = 5.66$, $HR = 2.508$, $p = 0.017$) [singular subject **and** plural subject] significantly **contributed** [simple past] to the observed differences. For treatments at 46 HPI, only initial pathogen **treatment** [subject – singular] **was** [verb – singular] a significant variable ($W = 7.62$, $HR = 0.205$, $p = 0.006$) (Fig. 4).

4. Discussion

During an infection, the desert **locust** [subject – singular] behaviourally **fevers** [verb – singular] by seeking out higher resting temperatures. In response to *M. acridum* infection, **a prolonged fever** [subject – singular] **occurred** [simple past] from 48 to 120 HPI. **This** [subject – singular] **is** [verb – singular] consistent with previous studies using *M. acridum* and other entomopathogens such as *Serratia marcescens* (Blanford and Thomas, 1999; Bunday et al., 2003). In contrast, only **a short-lived fever** [subject – singular] **was** [verb – singular] observed at 48 HPI during mycosis with *M. robertsii*. Analogous to the physiological fever experienced in mammals, behavioural **fever** [subject – singular] directly **impacts** [verb – singular] on pathogen growth and **can** [helping verb] 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** [subject – singular] **was** [verb – singular] reduced when locusts were prevented from fevers during mycosis. **Survival** [subject – singular] of locusts infected with *M. acridum* and allowed to thermoregulate freely **was** [verb – singular] comparable to similar studies using 5th instar and adult *S. gregaria* (Blanford and Thomas, 1999; Elliot et al., 2002). **Blanford and Klass** (2004) [subject – plural] **have** [verb – plural] previously assessed survival of locusts in the field under varied thermal environmental conditions. **The** reduced **survival** [subject – singular] of *M. acridum*-infected locusts restricted from fevers found here **was** [verb – singular] 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** [subject – singular] **is** [verb – singular] 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* [subject – singular] **was** [verb – singular] more virulent than *M. robertsii*. However, when **locusts** [subject – plural] **were** [verb – plural] allowed to thermoregulate, *M. robertsii* [subject – singular] **was** [verb – singular] more virulent, and *M. acridum*-infected **locusts** [subject – plural] **had** [simple past] survival patterns more similar to controls. Both **isolates** [subject – plural] **have** [verb – plural] similar thermal growth curves and **cease** [verb – plural] to grow above 40°C (Ouedraogo et al., 1997; Rangel et al., 2010). **This** [subject – singular], at least in part, **is** [verb – singular] likely to represent the different thermal regimes associated with each isolate. Survival **benefits** [subject – plural] **are** [verb – plural] only maintained for the duration of the fever response (Ouedraogo et al., 2004). The short-lived **fever** [subject – singular] of locusts infected with *M. robertsii* **is** [verb – singular] therefore likely to offer little advantage compared to that in locusts infected with *M. acridum*, where **fever** [subject – singular] **was**

[verb – **singular**] expressed throughout the photophase during the period of observation. **This** [subject – **singular**] indeed **appeared** [simple past] to be the case when survival **curves** [subject – **plural**] of the two cohorts of locusts **were** [verb – **plural**] compared.

Administration [subject – **singular**] of dtx A during infection with *M. acridum* **inhibited** [simple past] behavioural fever. **This** [subject – **singular**] **was** [verb – **singular**] most effective at 22 HPI, around the time at which **the fungus** [subject – **singular**] **penetrates** [verb – **singular**] the cuticle and **enters** [subject – **singular**] the haemolymph (Gunnarsson, 1988). At this point, early **stages** [subject – **plural**] of pathogen-recognition **occur** [verb – **plural**] in the haemolymph and **suppression** [subject – **singular**] of the immune system **may** [helping verb] provide the fungus with a greater survival advantage. **This** [subject – **singular**] **would** [helping verb] enable *M. acridum* to allocate resources to growth, rather than combating host defence. **An** inhibitory **role** [subject – **singular**] of dtxs on the insect immune system **is** [verb – **singular**] 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** [subject – **singular**] of such diverse immune defences **infers** [verb – **singular**] dtxs target early components of the pathogen recognition or immune response pathways. **Inhibition** [subject – **singular**] of behavioural fever reported here **may** [helping verb] likewise be targeted by dtxs as a component of the immune response. **Destruxins** [subject – **plural**] **are** [verb – **plural**] not the only secondary metabolites produced by *Metarhizium* spp. and attributed with a role in pathogenicity (Molnar et al., 2010). **Toxins** [subject – **plural**] which **are** [verb – **plural**] not well characterized or **are** [verb – **plural**] as yet unknown **may** [helping verb] also play a role in suppression of the insect immune system including behavioural fever. **Studies** [subject – **plural**] identifying genes such as those found for *M. acridum* mycosis of locust wings (He and Xia, 2009) and genome sequencing of *Metarhizium* isolates **will** [helping verb] help elucidate the array of secondary metabolites involved in pathogenicity (Gao et al., 2011; Molnar et al., 2010).

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

out for wild-type *M. acridum* by Klass et al. (2007), **would** [helping verb] be indicative of the most effective field conditions for application.

The amounts [subject – plural] of dtx injected in the present work **are** [verb – plural] likely to be greater than those determined in haemolymph during mycosis. There **are*** [verb – plural] no **figures** [subject – plural] available for the isolate and insect used here.

* In a sentence beginning with the phrase “there is” and “there are”, the verb agrees with the subject that follows (i.e., figures)

However, no direct **comparison** [subject – singular] **could** [helping verb] be made since hyphal **bodies** [subject – plural] in the haemocoel **are** [verb – plural] likely to provide locally high doses of dtx around aggregating **haemocytes** [subject – plural] which **could** [helping verb] not be equated with overall haemolymph concentrations. **The impact** [subject – singular] of sephadex beads coated with dtx beds on phagocytic haemocytes **illustrates** [verb – singular] this principle well (Huxham et al., 1989). Furthermore, metabolic **detoxification** [subject – singular] of dtxs by insect hosts of dtx-producing fungi means it **is** [verb – singular] difficult to quantify dtx levels *in vivo* (Soledade et al., 2002).

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