Cordycepin, a metabolite of *Cordyceps militaris*, reduces immune-related gene expression in insects

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**A B S T R A C T**

Hypocrealean entomopathogenic fungi (EPF) (Sordariomycetes, Ascomycota) are natural regulators of insect populations in terrestrial environments. Their obligately-killing life-cycle means that there is likely to be strong selection pressure for traits that allow them to evade the effects of the host immune system. In this study, we quantified the effects of cordycepin (3′-deoxyadenosine), a secondary metabolite produced by *Cordyceps militaris* (Hypocreales, Cordycipitaceae), on insect susceptibility to EPF infection and on insect immune gene expression. Application of the immune stimulant curdian (20 µg ml⁻¹, linear beta-1,3-glucan, a constituent of fungal cell walls) to *Drosophila melanogaster* S2r⁺ cells resulted in a significant increase in the expression of the immune effector gene *metchnikowin* compared to a DMSO-only control, but there was no significant increase when curdian was co-applied with 25 µg ml⁻¹ cordycepin dissolved in DMSO. Injection of cordycepin into larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) resulted in dose-dependent mortality (LC50 of cordycepin = 2.1 mg per insect 6 days after treatment). Incubating conidia of *C. militaris* and *Beauveria bassiana* (Hypocreales, Cordycipitaceae; an EPF that does not synthesize cordycepin) with 3.0 mg ml⁻¹ cordycepin had no effect on the numbers of conidia germinating *in vitro*. Co-injection of *G. mellonella* with a low concentration of cordycepin (3.0 mg ml⁻¹) plus 10 or 100 conidia per insect of *C. militaris* or *B. bassiana* caused a significant decrease in insect median survival time compared to injection with the EPF on their own. Analysis of predicted vs. observed mortalities indicated a synergistic interaction between cordycepin and the EPF. The injection of *C. militaris* and *B. bassiana* into *G. mellonella* resulted in increased expression of the insect immune effector genes lysozyme, IMPI and galericmycin at 72 h post injection, but this did not occur when the EPF were co-injected with 3.0 mg ml⁻¹ cordycepin. In addition, we observed increased expression of IMPI and lysozyme at 48 h after injection with *C. militaris*, *B. bassiana* and sham injection (indicating a wounding response), but this was also prevented by application of cordycepin. These results suggest that cordycepin has potential to act as a suppressor of the immune response during fungal infection of insect hosts.

1. Introduction

In order to successfully grow and reproduce in its host, a pathogen must evade the effects of the host immune system. This can be done through passive mechanisms (e.g. avoiding immune detection) or by actively interfering with host immune responses (Schmid-Hempel, 2009). Understanding the strategies used by pathogens for immune evasion provides valuable insights into the evolution of virulence in host-parasite relationships (Schmid-Hempel, 2009) but our present understanding of immune evasion in insect pathogens is not well developed.

Hypocrealean entomopathogenic fungi (EPF) (Sordariomycetes, Ascomycota) are obligately-killing parasites that infect a wide range of insects and are common in terrestrial ecosystems (Vega et al., 2009). Infection occurs by percutaneous growth of fungal spores into the haemocoel, from where the fungus starts to proliferate and consume host tissues, resulting in insect death (Chandler, 2016). In laboratory assays insect death usually occurs 3–7 days after infection, however this can take longer in a natural situation and varies between EPF species (Chandler, 2016). If environmental conditions are favourable, the
fungus grows back out of the insect cadaver to produce ascospores from fruiting bodies (for sexually reproducing forms) or vegetative conidia (for asexual reproduction), which are released into the environment to be acquired by naïve hosts. This particular life cycle, with transmission being dependent upon the pathogen killing its host, imposes a strong selection pressure for traits that confer high virulence in order to maximise pathogen fitness (Roy et al., 2006; Boomsma et al., 2014). Hypocrealean EPF produce a range of secondary metabolites during infectious growth (de Bekker et al., 2013), and there is experimental evidence that some of these disrupt cellular and systemic immune defenses, such as the destruxins, a group of cyclic hexadepsipeptides produced by Metarhizium species (Vilcinskas et al., 1997a, 1997b; Pal et al., 2007; Zimmermann, 2007a, 2007b). Indirect evidence on the anti-immune function of some other EPF metabolites comes from their use as immune suppressors in human medicine. Examples include cyclosporine, which is used to treat autoimmune diseases and to prevent rejection in organ transplantation, and which is a natural product from Tolypocladium inflatum (Hypocreales, Ophiocordycipitaceae) (Survase et al., 2011), as well as the immune-modulating drug fingolimod, used in the treatment of multiple sclerosis, and which is a synthetic derivative of myriocin, produced by Cordyceps/Isaria cicadae s.l. (previously known as Isaria sinclairii, currently undergoing taxonomic revision; Hypocreales, Clavicipitaceae) (Fujita et al., 1994; Strader et al., 2011; Kepler et al., 2017). The homologies between the innate immune systems of mammals and insects (Stokes et al., 2015) probably explains why immuno-suppressing metabolites from EPF are also able to affect the human immune system. The innate immune systems are sufficiently similar that insects are also being used increasingly as in vivo models of pathogen virulence in medical research (Sheehan et al., 2018).

Here, we present the results of a study on insect immune interference by cordycepin (3′-deoxyadenosine), a secondary metabolite produced by the teleomorphic hypocrealean EPF Cordyceps militaris (Hypocreales, Cordycipitaceae) (Cunningham et al., 1950; Shih et al., 2007; Tuli et al., 2014). This fungus occurs naturally in temperate and sub-tropical areas within Asia, Europe, and North and South America, although it is considered to be rare (Shrestha et al., 2012). It is a host specialist, causing natural infections predominantly in lepidopteran larvae and pupae (Shrestha et al., 2012). Cordycepin has been investigated previously for its pharmacological potential, particularly in connection with the use of C. militaris fruiting bodies as a traditional herbal medicine (Paterson, 2008). It has been reported to have anti-inflammatory (Kim et al., 2006; Jeong et al., 2010; Kondrashov et al., 2012; Zhang et al., 2014; Yang et al., 2015; Ashraf et al., 2019) as well as anti-tumour (Nakamura et al., 2006) and anti-angiogenic properties (Lu et al., 2014) in studies with mammalian cells, but little is known about its effect on insects. For the present study, we addressed the topic using Drosophila melanogaster (Diptera: Drosophilidae) cell culture and Galleria mellonella (Lepidoptera: Pyralidae) live caterpillars as experimental systems (Lemaitre et al., 1995; Mylonakis et al., 2005; Bergin et al., 2006; O’Neill, 2006; Ramaaro et al., 2012; Harding et al., 2013).

Firstly, we quantified the effect of cordycepin on the expression of immunity-related genes in a D. melanogaster S2r+ cell line treated with activators of the immune response. Secondly, we measured the survival of G. mellonella larvae, together with the expression of immunity-related genes, following injection with cordycepin on its own and in combination with C. militaris and Beauveria bassiana (Hypocreales, Cordycipitaceae), a related, asexually-reproducing EPF species that does not synthesize cordycepin (Xia et al., 2017). The aim was to provide new information on the effect of cordycepin on insect immune gene expression under the controlled conditions achievable using a cell-based assay, as well as the more biologically-complex conditions of a whole-animal environment.

2. Materials and methods

2.1. Experimental material

D. melanogaster S2r+ cells (Schneider, 1972; Rämet et al., 2001) were cultured in 15 ml cell culture flasks (VWR, Radnor, USA) with 12 ml of Schneider Drosophila Insect Medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich) and 10% penicillin-streptomycin (Sigma-Aldrich, St Louis, USA). Cell density was determined by trypan blue staining (Strober, 2001). Cells were subcultured when their density reached 1 × 10^6 cells ml⁻¹. All cells used in experiments were between passages 5–15.

Finally, non-diapausing G. mellonella larvae were purchased from Wazp Brand UK Ltd. (Yorkshire, UK). They were maintained at 15 °C in darkness and larvae between 200 and 300 mg were selected for experiments. Cordycepin (Sigma-Aldrich, St Louis, USA) was prepared as a stock solution (100 mg ml⁻¹ in DMSO; Thermo Fisher Scientific, Waltham, USA) and diluted with sterile 0.01% Triton X-100 (Merck, Darmstadt, Germany) wetting agent in DEPC-treated water plus 0.1 mg ml⁻¹ penicillin (G. mellonella, St Louis, USA) (Johnston, 2011).

Laboratory bioassays with entomopathogenic fungi were done using B. bassiana strain 433.99 and C. militaris strain ARSEF 11703 (Supplementary Table 1). Stock cultures of the strains were stored in cryopreservation (Chandler, 1994). Laboratory cultures were grown from stock cultures on Sabouraud Dextrose Agar (SDA; Thermo Fisher Scientific, Waltham, USA) slopes and maintained at 5 °C for up to six months. Subcultures for laboratory experiments were grown on SDA from slope cultures and incubated in darkness at 23 °C for 10–14 days. Conidia were harvested in sterile 0.01% Triton X-100 and suspensions filtered through milk filters (Lantor Ltd, Bolton, UK) to remove hyphal fragments. Conidia were counted using an Improved Neubauer haemocytometer and aliquots prepared at different concentrations as required.

2.2. Effect of cordycepin treatment on the response of D. melanogaster S2r+ cells to simulated immune challenge

D. melanogaster S2r+ cells were cultured as described above. Cells were collected by centrifugation at 1500 × g for 5 min at 4 °C, the supernatant removed and cells resuspended in Schneider Drosophila Insect Medium with 10% FBS. Aliquots (3 ml) were pipetted into each well of a 6-well plate (TPP tissue culture plate, Sigma-Aldrich, St Louis, USA) at a density of approximately 8 × 10^⁶ cells ml⁻¹. To determine the effect of cordycepin on gene expression, cells were treated with the immune response stimulants curdulan (linear beta-1,3-glucan; 20 µg ml⁻¹; CarboSynth Ltd., Compton, UK) or crude LPS (lipopolysaccharide; 20 µg ml⁻¹; Sigma-Aldrich, St Louis, USA), followed by addition of 25 µg ml⁻¹ (100 µM) cordycepin solution in DMSO, or DMSO only. An untreated control was also included. Curdulan is structurally similar to fungal cell wall polysaccharides and is used to stimulate an anti-fungal immune response (Kumar et al., 2009), whereas crude LPS (which also contains peptidoglycan) is found in the outer membrane of gram-negative bacteria and is used to stimulate an anti-(gram-negative) bacterial immune response (Tanji and Ip, 2005). Cells were incubated for 4 h at 25 °C, then RNA was extracted ahead of RT-qPCR (see below).

2.3. Effect of cordycepin treatment on the survival of G. mellonella larvae infected with the EPF C. militaris and B. bassiana

2.3.1. Dose-response bioassays

Dose response bioassays were done with cordycepin and EPF alone against G. mellonella larvae. For the cordycepin dose-response bioassay,
10 final instar *G. mellonella* larvae were cooled on ice for 5 min, then injected in the right front proleg with 30 μl cordycepin at 1.0, 1.8, 3.3, 6.0 or 11 mg ml⁻¹ (equivalent to 30, 55, 100, 180, and 330 μg per insect respectively, with a sham injection control) using a 0.3 ml microfine insulin syringe (BD, Franklin Lakes, USA). Where the suspension bled from larvae following injection, the larvae were discarded and not included in the experiment. Immediately after treatment, insects were placed individually in Petri dishes on damp filter paper, sealed with Parafilm, and maintained in darkness at 20 °C. Survival of the larvae was monitored every 24 h for seven days. The bioassay was repeated on three separate occasions, resulting in a total of 30 larvae in each treatment. The DRC package in R (Ritz et al., 2015) was used to estimate lethal concentration (LC) and lethal dose (LD) values at day 6. The EPF dose response bioassay was done in the same way, with final instar *G. mellonella* larvae being injected with 30 μl of conidia suspensions of *C. militaris* and *B. bassiana* at concentrations of 1 × 10⁷; 1 × 10⁶; 1 × 10⁵; 1 × 10⁴ and 1 × 10³ conidia ml⁻¹ (equivalent to 30, 300, 3 × 10³, 3 × 10⁴; 3 × 10⁵ conidia per insect respectively) in sterile 0.01% Triton X-100 (10 insects per treatment, sham injection controls, three independent repeats, LC and LD estimations at day 6).

2.3.2. EPF spore germination

The effect of cordycepin on EPF spore germination was measured as follows: for both *B. bassiana* and *C. militaris*, 100 μl conidia suspension (1 × 10⁷ conidia ml⁻¹ in sterile 0.01% Triton X-100) was combined with 30 μl of cordycepin (100 mg ml⁻¹) and made up to a final volume of 1 ml using Sabouraud Dextrose Broth (Sigma-Aldrich, St Louis, USA). This gave final concentrations of 1 × 10⁷ conidia ml⁻¹ and 3.0 mg ml⁻¹ (12 mM) cordycepin (equivalent to the LC₅₀ in the *G. mellonella* dose response bioassay). The mixture was incubated in darkness at 23 °C for 24 h, after which the germination of approximately 100 conidia was recorded by examination under a microscope. A conidium was considered germinated if the germ tube was longer than the length of the conidium. The experiment was performed on three occasions. Percentage conidia germination was analysed using a one-way ANOVA following a logit transformation (Warton and Hui, 2011) and statistical normality testing (Shapiro and Wilk, 1965) in SPSS (IBM Corp., 2016).

2.3.3. Effect of cordycepin application on *G. mellonella* susceptibility to EPF

A bioassay was done to measure insect survival following EPF infection supplemented with cordycepin. Batch of 20 final instar *G. mellonella* larvae were injected, as described above, with 30 μl conidia suspension of *B. bassiana* or *C. militaris* at concentrations of 3.3 × 10² and 3.3 × 10⁴ conidia ml⁻¹ (equivalent to 10 and 100 conidia per insect respectively) in 0.01% Triton X-100 with/without 3.0 mg ml⁻¹ cordycepin. Where the suspension bled from larvae following injection, the larvae were discarded and not included in the experiment. Assessment of larval survival was done as described above for 11 days and the experiment was repeated on three separate occasions, resulting in a total of 30 larvae for each treatment. For each insect batch, 10 individuals were used for survival analysis, while 5 individuals were removed at 0 h (t₀), 48 h and 72 h, and snap frozen in liquid nitrogen for RNA extraction and quantification of gene expression (Section 2.4). Changes in insect survival were visualised using a Kaplan-Meier estimator, and analysed using a Cox proportional-hazards regression model with replicate and treatment as factors, in which the median survival time (MST) of the insect populations of each treatment and their 95% confidence intervals were calculated, and pairwise comparisons were done using a log-rank χ² test (Cox, 1972; IBM SPSS Statistics Version 24; Bewick et al., 2004). The outcome of combining cordycepin with *B. bassiana* or *C. militaris* on total percentage mortality (synergism, antagonism, or additive effect) at day 6 and day 11 was investigated using the fractional product method for combination treatments, where the effect of the combination is given as (1 – x), where X = (1 – A)/(1 – B) and where A = the proportional effect of agent A on its own, and B is the proportional effect of agent B (Webb, 1963).

2.4. Quantification of expression of insect immune-associated genes

RNA was extracted from *D. melanogaster* S2r+ cells using the ReliaPrep RNA Cell MiniPrep system (Promega, Madison, USA) following manufacturer’s instructions. For RNA extraction from *G. mellonella*, individual snap frozen larvae were ground in liquid nitrogen using an autoclaved mortar and pestle. RNA was extracted from 50 mg of material using the phenol:chloroform method with Tri-reagent (Sigma-Aldrich, St Louis, USA) then treated with DNase I (Sigma-Aldrich, St Louis, USA) following manufacturer’s instructions. After washing with 75% ethanol, air dried RNA pellets were resuspended in 50 μl DEPC-treated water and stored at −80 °C. RNA concentrations were measured using a NanoDrop® ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). cDNA was synthesized using Superscript kits (Thermo Fisher Scientific, Waltham, USA); Superscript III was used to reverse transcribe *D. melanogaster* RNA (100 μg) and Superscript II was used for *G. mellonella* RNA (500 μg), both with random hexamers. Manufacturer’s instructions were followed, with the exception that 0.5 μl (100 units) SuperScript III was used for reactions. The expression of insect immune associated genes was quantified by RT-qPCR using a Lightcycler 480 (Roche Holding AG, Basel, Switzerland). For *D. melanogaster* S2r+ cells, expression levels were quantified for genes encoding the ribosomal protein RP49 (used as the reference), and the antimicrobial peptides (AMPs) metchnikowin and attacin A. For *G. mellonella*, expression levels were quantified for genes encoding the following proteins: the ribosomal protein S7e (reference); the AMPs gallerimycin and galiomicin; lysozyme; and the insect metalloproteinase inhibitor (IMPI). Gene expression in *G. mellonella* was measured for larvae injected with EPF +/− exogenous cordycepin, as well as for sham injections and un.injected controls. Primers for all genes are shown in Table 1. SensiFAST SYBR No-ROX (Bioline, London, UK) was used for RT-qPCR, each reaction contained: 5 μl SYBR Green, 2 μl DEPC treated water, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM) and 1 μl cDNA. The conditions were: 95 °C 2 min, 40 × (95 °C 5 s, 60 °C 10 s and 72 °C 20 s). The comparative C₇ method (also known as the 2ΔΔC₇ method) (Schmittgen and Livak, 2008) was used to calculate fold changes in gene expression compared to untreated samples with correction for the internal control. Each gene of interest was analysed independently. For *D. melanogaster*, data on relative gene expression were tested for normality (Shapiro and Wilk, 1965) before being subject to ANOVA, while for *G. mellonella*, the data were tested for normality and then analysed using Kruskal-Wallis H tests (IBM SPSS Statistics Version 24). Where overall significant differences were observed, post-
hoc analysis was performed using a Tukey HSD post hoc test (following ANOVA) or the Dunn-Bonferroni post hoc method (following Kruskal-Wallis tests).

3. Results

3.1. Effect of cordycepin treatment on gene expression in D. melanogaster S2r+ cells following immune challenge

The relative expression of the immune effector genes metchnikowin and attacin was quantified in a D. melanogaster S2r+ cell line treated separately with two activators of the immune response, curdlan and LPS, and with and without the addition of 25 µg ml⁻¹ cordycepin (Fig. 1). There was significant variation among treatments in the relative expression for both metchnikowin (F5,17 = 5.93, P < 0.01) and attacin A (F5,17 = 3.48, P < 0.05). Application of curdlan significantly elevated the mean relative expression of metchnikowin compared to the DMSO-only control or DMSO plus cordycepin (Tukey HSD, ν = 5, P < 0.05; ANOVA, Tukey HSD post hoc tests). The mean germination (back-transformed) of Conidia populations was 75% and 79% at 0 and 3 mg ml⁻¹ of cordycepin respectively, while for C. militaris conidia populations was 75% and 79% at 0 and 3 mg ml⁻¹ of cordycepin respectively, while for C. militaris conidia it was 82% and 79% respectively (Fig. 4).

3.2. Effect of cordycepin treatment on the survival of G. mellonella larvae infected with C. militaris and B. bassiana

In this set of experiments we investigated whether application of exogenous cordycepin would affect the susceptibility of G. mellonella larvae to fungal infection and impact on the expression of host immune genes. We used a low dose of cordycepin that did not inhibit fungal activity and did not cause excessive insect mortality on its own.

3.2.1. Dose-response bioassays of B. bassiana and C. militaris against G. mellonella larvae

Larvae of G. mellonella were treated with either EPF or cordycepin to establish the impact of these treatments individually on insect survival. There was a positive relationship between G. mellonella mortality and dose of cordycepin applied (Fig. 2, Weibull type 1 model), the LC₅₀ of B. bassiana and LD₅₀ of C. militaris were also calculated on day 6 after treatment and found to be 2.91 (±0.35) mg ml⁻¹, 6.96 (±1.03) mg ml⁻¹, 87.95 (±10.79) µg per larva and 209.34 (±30.70) µg per larvae, respectively. Individual larvae receiving a lethal dose of cordycepin became distinctly grey in colour between 24 and 48 h prior to death (Supplementary Fig. 1) and their movement declined markedly. After death, cadavers turned black and their abdomens were fluid-filled. G. mellonella larvae were susceptible to lethal infections by both B. bassiana and C. militaris (Fig. S1; estimated B. bassiana LC₅₀ (day 6) = 3.6 × 10⁵ (±9.4 × 10⁵) conidiain ml⁻¹, LD₅₀ = 1.1 × 10⁵ (±2.8 × 10⁵) conidia per insect; C. militaris LC₅₀ = 4.0 × 10⁴ (±9.1 × 10⁴) conidia ml⁻¹, LD₅₀ = 1.2 × 10⁵ (±2.8 × 10⁵) conidia per insect; two-parameter log-logistic model).

3.2.2. EPF conidia germination

Conidia of C. militaris and B. bassiana were incubated with cordycepin to identify any inhibitory effects on germination. There was no significant effect of cordycepin treatment (3 mg ml⁻¹) on the numbers of B. bassiana and C. militaris conidia germinating in vitro after 24 h (F₅,₁₁ = 1.045, P > 0.05). The mean germination (back-transformed) of B. bassiana conidia populations was 75% and 79% at 0 and 3 mg ml⁻¹ of cordycepin respectively, while for C. militaris conidia it was 82% and 79% respectively (Fig. 4).

3.2.3. Effect of cordycepin application on G. mellonella susceptibility to fungal infection

A laboratory bioassay was done to quantify the effects on G. mellonella survival of co-applying 3 mg ml⁻¹ cordycepin with C. militaris or B. bassiana at doses of 10 and 100 conidia per insect. Mortality of G. mellonella larvae in the sham injection control was 3.3% at 11 days post injection (dpi) (Supplementary Table 2). Injection of larvae with 3 mg ml⁻¹ cordycepin resulted in a mortality of 13.3%, which was in keeping with the results from the earlier cordycepin dose response bioassay (Fig. 2) but was not statistically significantly different from the sham injection control (Supplementary Table 2). Compared to injection of EPF on their own, co-injection of 3 mg ml⁻¹ cordycepin with

![Fig. 1. Expression of AMPs in D. melanogaster S2r+ cells in response to cordycepin treatment. S2r+ cells (three biological replicates) were treated with DMSO (dark grey bars) or DMSO plus 25 µg ml⁻¹ (100 µM) cordycepin (light grey bars). Immune challenge was simulated using 20 µg ml⁻¹ LPS or curdlan. Cells were sampled 4 h after treatment. Fold changes in gene expression were calculated using the 2⁻ΔΔCT method (Schmittgen and Livak, 2008) compared to untreated samples with correction for the internal control (RP49). Relative expression of A) metchnikowin and B) attacin A is shown. Error bars show ± SEM and different lowercase letters indicate significant differences (p < 0.05; ANOVA, Tukey HSD post hoc tests).
B. bassiana or C. militaris caused a significant decrease in median survival time of c. 24 h in all cases (Supplementary Table 2, log rank chi squared > 3.841, P < 0.05; also see survival curves, Fig. 5) and this was reflected by an increase in the hazards ratio (Supplementary Table 2). A dose of 10 conidia per insect of C. militaris resulted in 36.7% mortality at 11 dpi, meaning that median survival time could not be estimated (Supplementary Table 2). At day 6 the predicted mean mortality of B. bassiana + cordycepin combination, calculated from the fractional product of B. bassiana and cordycepin mortality individually, was 0% at a dose of 10 conidia per insect (observed value = 26.7%) and 46.7% at a dose of 100 conidia per insect (observed value = 80%). At day 11 the predicted mean mortality of the C. militaris + cordycepin combination was 45.1% at a dose of 10 conidia per insect (observed value = 80%), and 65.3% at a dose of 100 conidia per insect (observed value = 80%). Both the results on days 6 and 11 after treatment indicate that there was a synergistic effect of the combination treatment with EPF and cordycepin (Webb, 1963).

3.3. Effect of cordycepin treatment on the expression of immune-related genes in G. mellonella larvae infected with B. bassiana and C. militaris

In order to provide information on the possible mechanism by which cordycepin affects fungal infection, the expression of four G. mellonella immune-related genes (galiomicin, gallerimycin, IMPI and lysozyme) was quantified at 48 h and 72 h in larvae injected with B. bassiana or C. militaris.
Fig. 5. Survival curves of *G. mellonella* treated with cordycepin and EPF. Survival of *G. mellonella* larvae injected with 10 or 100 *B. bassiana* conidia or *C. militaris* conidia. Solid lines show observed mortalities, those lines ending in ‘+’ indicate censored populations. Dashed lines indicate the expected population decline (shaded area illustrates the standard error) estimated by fitting a binomial generalized logistic model. Pale grey lines indicate treatment with cordycepin (3 mg ml$^{-1}$) and dark grey with a DMSO control. N = 30 per treatment. P values are the result of a log-rank test.
C. militaris \(+/-\) cordycepin (Fig. 6). The experiment also included a sham injection control and an untreated (=un-injected) control. Pairwise comparisons (Dunn-Bonferroni method) showed that, at 48 h, injection with C. militaris, B. bassiana, and sham injection resulted in a significantly larger increase ($P < 0.05$) in relative expression of IMPI and lysozyme compared to the untreated control. At 72 h, injection with C. militaris and B. bassiana resulted in a significantly larger increase ($P < 0.05$) in relative expression of gallerimycin, IMPI, and lysozyme compared to the untreated control, but there was no significant difference between sham injection and the untreated control for any of the four genes ($P >$
the anti-fungal elicitor curdlan resulted in increased expression of following curdlan treatment. Specifically, while immune challenge with metchnikowin effect on expression of the anti-fungal AMP gene (Fig. 6). Compared to injection between 2-fold to 7-fold reduction in relative gene expression depending on the EPF species and immune gene (De Gregorio et al., 2002; Dong et al., 2012; Yi et al., 2014). Our finding of significantly increased attacin A expression in the D. melanogaster S2r+ cell assay in the curdlan vs. cordycepin-only treatment, provides some tentative support that attacin A expression is responsive to fungal infection.

The inhibitory effects of cordycepin on the anti-fungal insect immune response was further supported by the results from our G. mellonella experiment, where application of cordycepin prevented increased expression of different immune effector genes in response to fungal infection, and resulted in faster host insect death. Injection of G. mellonella larvae with B. bassiana, C. militaris, or a sham injection, resulted in increased expression of lysozyme, IMPI and gallerimycin (but not gallerimycin) in response to B. bassiana and C. militaris injection, but there was no elevated immune gene expression in response to sham injection compared to the untreated control, suggesting that the gene expression response to wounding was short lived. Applying exogenous cordycepin alongside B. bassiana or C. militaris, or as an addition to the sham injection, dampened down the expression of these immune genes so that relative expression was no different from the untreated control. Gallerimycin and gallerimycin are both anti-fungal defensins (Schuhmann et al., 2003; Lee et al., 2004; Langen et al., 2006) synthesized via the Toll pathway in response to EPF infection (Vertyporokh and Wojda 2017). Lysozyme has both bacterial and anti-fungal activity (Jolles and Jolles, 1984; Wojda et al., 2009; Sowa-Jasite et al., 2016), while IMPI is a G. mellonella-specific inhibitor of EPF metalloproteinases that hydrolyses insect proteins in the cuticle and haemolymph (Vilcinskas and Wedde, 2002; Altincicek et al., 2007; Wedde et al., 2007), and is itself a target for EPF proteinases synthesized as a fungal countermeasure to the G. mellonella immune response (Mukherjee and Vilcinskas, 2018). Expression of IMPI has been shown previously to be up-regulated within 48 h of fungal infection (Vertyporokh and Wojda, 2017). Expression of both IMPI and lysozyme in G. mellonella is reported to be upregulated by a range of stimuli including fungal and bacterial infection (Vertyporokh and Wojda, 2017), metamorphosis and wounding (Griesch et al., 2000; Altincicek and Vilcinskas 2006; Vilcinskas, 2019). The signalling pathway(s) leading to IMPI and lysozyme expression in G. mellonella have yet to be fully elucidated, although they are likely to be regulated by Toll, Imd or a related pathway as their transcription is controlled by c-Rel proteins Relish, DIF and Dorsal (Altincicek and Vilcinskas, 2006; Vilcinskas, 2019).

We observed no upregulation of gallerimycin in response to fungal infection in G. mellonella, which may have been due to the route of infection (injection rather than topical application) or because gene expression was measured using whole insects rather than specific tissues, such as the fat body, which is a site of AMP synthesis (Vertyporokh and Wojda, 2017). We chose injection as the way to deliver cordycepin to G. mellonella as it mimics the normal route of exposure, since host tissues are exposed to it after fungal hyphae have grown into the insect haemocoel. Injection of EPF also allowed us to precisely control the dose of pathogen, and to ensure that cordycepin and EPF were applied at the same time and location.

To date, studies on the effects of cordycepin have focused on the mammalian innate response rather than the insect immune system (for example see Chu and Edelman, 1972; Penman et al., 1970;Kim et al., 2006; Jeong et al., 2010; Kondrashov et al., 2012; Ren et al., 2012; Ashraf et al., 2019). The close similarities between aspects of the mammalian innate immune system and insect immunity - such as the homology between TLR (mammal) and Toll (insect) immune signalling (Sheehan et al., 2018) - would explain why cordycepin is able to affect both mammalian and insect immune responses. Cordycepin treatment is reported to reduce inflammatory mRNAs in primary human airway smooth muscle cells and mouse macrophage cells treated with an
insect tissue type, and whether fungal growth is infectious or sapro-
phagous to suppress the expression of different antimicrobial effectors is
(Lepidoptera) and, in the same study, a crude destruxin preparation was
produced by non-specialist EPF has been shown to vary between species,
beneficial for fungal fitness. The array of secondary metabolites pro-
duced during fungal pathogenicity and host immune defences (Boo-
manna et al., 2014; Mukherjee and Vilcinskas, 2015), as well as practical
applications such as the selection and development of EPF strains for
biological pest control or the identification of secondary metabolites as
novel pesticides, efficacy enhancing agents, or pharmaceuticals (Butt
et al., 2016).

Declaraton of Competing Interest

The authors declare that they have no known competing financial
interests or personal relationships that could have appeared to influence
the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.
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