Research Article
Immune Response of Mormon Crickets That Survived Infection by Beauveria bassiana

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Beauveria bassiana (Fungi: Ascomycota) is an entomopathogenic fungus that serves as a biological control agent of Mormon crickets Anabrus simplex Haldeman (Orthoptera: Tettigoniidae) and other grasshopper pests. To measure the dose-dependent response of Mormon crickets to fungal attack, we applied Beauveria bassiana strain GHA topically to adults using doses of $5.13 \times 10^4$ to $1.75 \times 10^6$ conidia in sunflower oil, with oil only as a control. After three weeks, we assessed the survivors’ hemolymph for fungal cells, active phenoloxidase (PO), and lysozyme. Mortality increased and body mass of survivors decreased with conidial dose. Survivors’ PO activity was elevated to the same level independent of dose. Those with fungal cells visible in their hemolymph did not differ in PO activity from those with clear hemolymph. We conclude that circulating PO may be an important enzymatic defense against Beauveria infection and that it is associated with attempted clearing of Beauveria blastospores and hyphae from Mormon cricket hemolymph.

1. Introduction

Nomadic insects risk contact with fungal pathogens [1]. Mormon crickets, a long-horned grasshopper or katydid, form bands and march across western United States grasslands seeking food, salt, and oviposition sites (Figure 1, [2, 3]). Wingless, they must walk, which increases the risk of contacting insect-pathogenic ascomycetous fungi, such as Beauveria spp. and Metarhizium spp., on plants or soil [4]. These fungal pathogens occur naturally, but some strains, such as the commercial Beauveria bassiana GHA, may be applied artificially as control agents.

The ability of the fungus to infect an insect depends on its ability to adhere and penetrate the exoskeleton, resist the insect’s hemolymph-borne defenses, and grow rapidly [5]. The conidium adheres to the cuticle and germinates to penetrate the exoskeleton with a combination of mechanical pressure and a cocktail of lytic enzymes. The insect may respond to the wounding with local induction of the phenoloxidase (PO) cascade, resulting in production of toxic quinones and cuticular melanization. Following penetration into the hemolymph, the fungus grows as a yeast-like blastospore or as short lengths of vegetative hyphae. Humoral defenses of insects to pathogenic fungi have only been investigated in a handful of species. Metarhizium infection may result in declining hemolymph protein and PO titres over the course of the infection until death (Schistocerca gregaria [10], Locusta migratoria [6]) whereas Beauveria infection increases active PO levels (Melanoplus sanguinipes [11], Spodoptera exigua [12]). Lysozyme activity may decline (Schistocerca gregaria [10]) or
remain unchanged (*Spodoptera exigua* [13]). In this paper, we investigate circulating PO and lysozyme titres in adult Mormon crickets that have successfully defended themselves against invasion from topically applied *Beauveria bassiana* strain GHA. On rangeland and crops, control agents are frequently not applied until Mormon crickets have reached the adult stage because the public demand for control is greatest when Mormon crickets have banded together and migrated from natal sites into habitats where they interfere with human activities.

## 2. Materials and Methods

### 2.1. Fungal Conidia

The *Spodoptera exigua* remain unchanged ([13]). In this paper, we investigate circulating PO and lysozyme titres in adult Mormon crickets that have successfully defended themselves against invasion from topically applied *Beauveria bassiana* strain GHA. On rangeland and crops, control agents are frequently not applied until Mormon crickets have reached the adult stage because the public demand for control is greatest when Mormon crickets have banded together and migrated from natal sites into habitats where they interfere with human activities.

### 2.2. B. Bassiana Dose Response

Adult Mormon crickets were collected at Lodge Grass, Montana on July 17, 2007, and fungal treatments were topically applied on July 24 (1st replicate) and July 25 (2nd replicate) to the base of the first leg, including the following fungal doses suspended in 1 μl sunflower oil: 1.75 × 10⁶, 1.07 × 10⁶, 3.54 × 10⁵, 1.13 × 10⁵, or 5.13 × 10⁴ conidia/μl *B. bassiana* strain GHA or a control treatment of only sunflower oil. Survivorship was measured over 21 days at 28°C.

### 2.3. Immunity Assays and Total Protein

After three weeks, we drew hemolymph from the surviving adults (five males and five females for each treatment, fewer if there were not enough survivors) to assess spontaneously active PO, lysozyme-like activity, and total hemolymph protein. We measured the body mass of each cricket to the nearest mg with an Ohaus microbalance (model AV53) and then punctured the arthrodial membrane at the base of the hind leg of each insect with a 26 gauge hypodermic needle so that it exuded hemolymph. A total of 14 μL of hemolymph was collected into a capillary tube, with a second puncture performed when necessary. For assays of PO activity and total hemolymph protein, the hemolymph was diluted 1:50 with phosphate buffered saline (PBS) solution and frozen at −20°C. An additional 10 μL hemolymph diluted 1:10 with PBS was stored at −20°C for subsequent measuring of lysozyme activity. For ten insects, we did not collect sufficient blood for all of the tests.

To measure PO activity, we followed the protocol of Wilson et al. [8]. Samples of thawed hemolymph diluted in PBS were centrifuged (4°C, 10,300 rpm for 10 minutes) and activated with 10 mM dopamine solution. The plate was loaded into a temperature-controlled BioTek microplate reader (25°C), and absorbance at 492 nm was read between 5 and 15 minutes. If sample absorbance was linearly related with time, we calculated mean $V$ (change in absorbance min⁻¹). One unit PO activity per ml hemolymph is defined as the amount of enzyme resulting in a 0.001 increase in absorbance.

To measure lysozyme-like antibacterial activity, a turbidimetric method was used, following the protocol of de Azambuja et al. [14]. Thawed and PBS-diluted hemolymph was added to a well with suspended gram-positive bacteria cells *Micrococcus lysodeikticus* (Worthington). Clearing of the well was compared to a serial dilution of egg-white lysozyme (Sigma) added to the bacteria suspension. The plate was loaded into a temperature-controlled Biotech microplate reader (25°C), and absorbance at 450 nm was read between 10 and 30 minutes. If the sample absorbance was linearly related with time, we would calculate mean $V$. When sample activity fell below 6.5 μg ml⁻¹, the sample was excluded because the standards showed that the data were unreliable when samples were this weak.

We measured total hemolymph protein in mg protein ml⁻¹ hemolymph with a Total Protein Kit, Micro (Sigma) compared to a serial dilution of the human albumin standard.

### 2.4. Verifying Infection

An additional 10 μL of hemolymph collected as described above was smeared on a slide and stained with a drop of lactofuchsin. Hemolymph samples were scanned at 400x, using dark-field, phase-contrast microscopy, for hyphae and blastospores.

### 2.5. Statistical Analyses

To analyze the *B. bassiana* dose response data, we combined the data from both replicates because Fisher’s Exact Tests indicated no significant differences between the replicates at each dose. The combined data...
Table 1: Pathogenicity of *Beauveria bassiana* strain GHA for adult *Anabrus simplex* based on mortalities 21 days after topical application.

<table>
<thead>
<tr>
<th>LD50 (conidia/insect)</th>
<th>95% Confidence Limits (conidia/insect)</th>
<th>Slope (S.E)</th>
<th>Chi-Square (P)*</th>
<th>g**</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.46 × 10^5</td>
<td>3.97 × 10^5–6.125 × 10^5</td>
<td>0.885</td>
<td>6.745 (.08)</td>
<td>0.144</td>
</tr>
</tbody>
</table>

*Chi-square of heterogeneity: measures goodness of fit to the weighted regression line with P > .05 indicating a good fit of the data to the line. D.F. = 5
**g is the index of regression significance.

were then subjected to probit analysis using LDP Line (LDP Line, 2000 by Ehab Mostofa Bakr, Cairo, Egypt). Lysozyme and log10-transformed PO were normally distributed. Applying ANCOVA, we covaried the dependent variables with body mass and tested them for effects of replicate, sex and fungal dose (sample sizes in order of dosage from highest to lowest: n = 2, 8, 9, 10, 10, and 10 for the 1st replicate and n = 3, 5, 8, 6, 9, and 10 for the 2nd). Body mass was not a significant covariate, and so here we report the results from the three-way ANOVA’s. Only for the males did the total protein meet the assumptions for parametric statistical analyses, and so we applied nonparametric statistics to data for the females.

Data for PO and total protein were normally distributed after log_{10} transformations. Lysozyme activity was normally distributed after squaring the data. Applying ANCOVA, we covaried the dependent variables with body mass and tested them for effects of sex and fungal treatment. However, body mass was not a significant covariate, and so we simplified the analysis and reported the two-way ANOVA’s.

3. Results

Mortality at 21 days ranged from 22% to 80% and increased with the dose of *B. bassiana* applied to the cuticle (Table 1) with an LD50 estimate of 6.46 × 10^5 conidia per insect.

For survivors, mean body masses of replicates were significantly different (P = .038), and those for all treatments except one were significantly less than that for controls, but there was no difference in body mass among *B. bassiana* doses (Figure 2(a)). Log PO differed significantly between replicates and dose (P = .0015 and P = .0048, resp.) whereas it did not differ between the sexes (P = .80). In a post hoc comparison among the means, Mormon crickets treated with *B. bassiana* had greater PO activity than uninfected controls, but none of the fungal treatments differed from one another (Figure 2(b)). The second replicate also had significantly greater lysozyme activity than the first (P = .030) whereas sex and dose did not have significant effects (P = .81 and P = .57, resp.). Within males, total protein was proportional to body mass (P < .0001), and insects in the second replicate had significantly greater total protein than those in the first (P = .0025, resp.), but fungal treatment was not a significant factor affecting total protein (P = .635). Females in the second replicate also had significantly greater total protein than those in the first replicate (Wilcoxon test, S = 423, z = 2.02, P = .043), but fungal treatment was not a significant factor affecting total protein within replicates (P > .60).

4. Discussion

Mormon crickets responded to *B. bassiana* infection with an increase in PO. *Beauveria* infection also increased active PO levels in the grasshopper *Melanoplus sanguinipes* and the army cutworm *Spodoptera exigua* [12]. Gillespie and Khachatourians [11] found that after topical application of 10^9 conidia to *M. sanguinipes*, PO levels increased 3.8 times in males peaking at 3 days postinfection and 8.3 times in females peaking on the first day postinfection. In *M. sanguinipes* after 5 days, PO levels had returned to near control levels in males, but in females remained more than twice that of controls. Our applied doses were lower, and more of the Mormon crickets survived the application. At 21 days, PO levels remained higher in *Beauveria*-treated Mormon crickets relative to controls. We did not observe a difference in PO levels between the sexes for either controls or those that survived fungal application. Surprisingly, PO titres of *Beauveria*-treated survivors were independent of the dose applied.
Total circulating protein concentrations did not differ between treatments in males or females. In *Melanoplus sanguinipes*, protein concentrations of males and females peaked 30% above that of controls within three days of infection, but returned to the same level as controls by day five post infection [11].

The second replicate had higher PO, lysozyme, and total protein titers than the first. Adults were collected from the same location on the same day and treated only a day apart to make replicates as similar as possible, and thus the reason for these differences is not known. Body mass of individuals did not differ significantly between control groups (*n* = 20, *P* = .38), and so individuals in the second replicate were probably in no better overall condition to defend against the fungus than the first. Indeed, the average mass of the first replicate was 6% greater than that of the second replicate—the opposite of what one would expect if condition were a factor. *Beauveria*-treated individuals lost on average 17% of their mass relative to controls. Reduced food consumption is the most likely cause. *Schistocerca gregaria* eats less when infected with *Metarhizium* [15], and *Manduca sexta* stops feeding altogether [16]. However, an increase in metabolism with infection could also increase mass loss. Metabolic rate might increase because the Mormon cricket is fending off the infection or as a result of the contribution of the growing fungus. Reduced nutrient absorption from the gut or greater water loss might also contribute to mass loss and warrant further study.

PO activity of survivors with fungal cells visible in their hemolymph did not differ significantly from those with clear hemolymph (*n* = 57 fungus absent, *n* = 9 fungus present, Welch ANOVA *F* = 0.06, d.f. = 1, 9, *P* = .81, Figure 3). We conclude that circulating PO may be an important enzymatic defense against *Beauveria* infection and that it is associated with attempted clearing of *Beauveria* blastospores and hyphae from the hemolymph of Mormon crickets.

*Beauveria bassiana* infection did not affect lysozyme activity in the Mormon crickets. Hence, elevation of PO did not result in an elevation of antibacterial activity in an all-or-none manner. Lysozyme activity declined with *Beauveria* infection in the desert locust *Schistocerca gregaria* [10] but remained unchanged in the army cutworm *Spodoptera exigua* [13].

In some Mormon cricket bands, migrating individuals seek protein [3], and protein ingestion is associated with an increase in PO activity [17]. Thus, protein deficiency evident in migratory bands is also likely to result in greater susceptibility to and more efficacious application of *B. bassiana* GHA.

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**References**


