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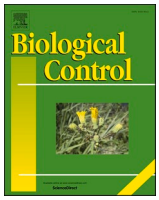

Understanding how diet and temperature affect survival and subsequent sporulation in a major rangeland grasshopper pest, *Melanoplus sanguinipes*, infected with the entomopathogenic fungus, *Metarhizium robertsii*

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HIGHLIGHTS

- We analyzed how *Metarhizium* inoculation and febrile conditions impacted intake targets in *M. sanguinipies*.
- All treatments selected the same carbohydrate-biased IT of 1:2 protein:carbohydrate (p:c).
- Grasshoppers with access to febrile conditions were able to completely rescue themselves from inoculation.
- Inoculated grasshoppers in the carbohydrate-biased and protein-biased treatment groups survived longer than the balanced diet.
- Post-mortem *Metarhizium* growth was greatest in the balanced diet treatment and minimal on grasshoppers eating the other diets.

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ABSTRACT

Behavioral fever is well-described in insects as an effective response to pathogens, but recent research also shows that the balance of macronutrients is important. Australian plague locusts (*Chortoicetes terminifera)* infected with *Metarhizium acridum*, a *fungal entomopathogen,* had longer survival by increasing carbohydrate and decreasing protein consumption. Our research tested the effects of *Metarhizium robertsii* (strain DWR2009) on the dietary macronutrient balance (Intake Target, IT) of the migratory grasshopper (*Melanoplus sanguinipes*, one of the most pestiferous grasshoppers in the United States), with and without elevated temperatures, to determine if pathogens significantly influence diet selection in this species. We also tested the effects of diet on survival under a *M. robertsii* inoculation. We found no significant difference in the ITs across all treatment groups; all treatments selected the same carbohydrate-biased IT of 1:2 protein:carbohydrate (p:c). In the prescribed diet experiments, inoculated grasshoppers from both the carbohydrate-biased (7p:35c) and protein-biased (35p:7c) diet treatment groups survived longer than those fed the balanced (21p:21c) diet. However, grasshoppers with access to elevated temperatures were able to completely rescue themselves from the pathogen. In correlation with our results, post-mortem *Metarhizium* growth was greatest on grasshoppers fed the balanced (21p:21c) diet and minimal on grasshoppers eating the other two diets. Eating the balanced 21p:21c diet either did not support the host to mount an effective immune response, provided a nutritionally optimal environment for pathogen growth, or both. Eating a protein-biased diet potentially supported an effective immune response, whereas eating a carbohydrate-biased diet starved the pathogen of protein and/or supported an immune response via different pathways.

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1. Introduction

One of the big challenges that agriculture sectors globally face every year is a variety of pests decimating crops. With more people looking to move away from traditional chemical pesticides to manage agriculture pests, biological pesticides (biopesticides) offer potential management alternatives, which can sometimes decrease long-term non-target effects relative to broad-spectrum pesticides (Peveling et al. 2003, Zimmermann 2007a,b, Chandler et al. 2011, Maute et al., 2017, and Seiber et al 2018). Orthoptera species are good candidates for biopesticide use because they can be major rangeland and cropland pests in many countries, several of which have annual population management programs. For example, biopesticides in the form of fungal pathogens are currently used effectively in Australia (Hunter 2004), Mexico (Poot-Pech and García-Ávila 2019), and China (Zhang and Hunter 2017). For contrast, in the U.S., by late July of 2021, the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) treated 805,000 acres for grasshoppers or Mormon crickets using insecticides (USDA-APHIS, 7/27/2021), but the USDA and land managers are very interested in moving to more environmentally friendly methods of population management, with decades of research invested into developing viable biopesticides that are indigenous to the U.S. (Cunningham and Sampson 1996-2000, USDA-APHIS, 7/27/2021 aphis.usda.gov, Gardner and Thomas 2002). While biopesticides are viable methods for management under some circumstances, they are still not used as much as traditional pesticides because they are generally less effective in terms of efficacy and mortality speed (Copping and Menn 2000). The decreased effectiveness is due to grasshoppers' natural ability to fight infections, especially through behavioral fever, but also encapsulation, melanization, and shifting diet (Carruthers et al. 1992, Blanford and Thomas 1999 and 2000, Jaronski 2010, Graham et al. 2014)). For example, biopesticide application is typically recommended when field temperatures are low and during cloudy weather to preclude grasshoppers from implementing behavioral fever (Jaronski 2010).

Two common biopesticides used in grasshopper management are fungal entomopathogens, *Metarhizium acridum* (Driver, Milner, J.F. Bisch., Rehner & Humber, 2009) and *Beauveria bassiana* (Bals.-Criv. Vuill., 1912). These fungi have a percutaneous rather than peroral route of infection. The standard mode of infection for fungal pathogens follows this order: adhesion of the conidia (fungal spore) to the insect cuticle, germination, appressorium formation (specialized cell that uses turgor pressure to penetrate the host's cuticle), penetration, colonization of the hemolymph, and, after the insect has died and under permissive conditions, emergence from the cadaver and sporulation (Vega et al. 2012, Keswani et al. 2013, and Aw and Hue 2017). Once cuticle penetration occurs and the fungus penetrates into the hemocoel of the host, it will use the nutrients available in the hemolymph to reproduce and grow, producing secondary metabolites. Pathogens that reproduce and grow quickly tend to kill their hosts with secondary metabolites, whereas those that reproduce and grow slower are thought to kill the host by depleting nutrients and/or causing structural damage to tissues in the organism, (Vega et al. 2012). Both *B. bassiana* and *Metarhizium* spp. produce secondary metabolites that vary in toxicity to insects. *Beauveria* produces oxalic acid, beauvericin, bassianolide, and hydroxybenzoquinone, some of which contribute to pathogenicity. *Metarhizium* spp. produce destruxins, which are cyclic depsipeptides that act on the immune functions of insects and cause cellular damage (Vega et al. 2012). In some cases, these destruxins can negate the effects of phagocytosis, cellular encapsulation, and in some cases behavioral fever, effectively preventing the host from mounting a defense against the infection and, thus, the host eventually succumbs to the fungus (Hunt and Charnley 2011, Vega et al. 2012, Aw and Hue, 2017).

Elevating body temperature above the upper tolerance of a pathogen (inducing a fever), is a prominent immune response for several insect taxa. Many taxa of flies, and grasshoppers can induce behavioral fever by actively thermoregulating to elevate their body temperature through behaviors, such as basking in direct sunlight (Carruthers et al. 1992). Many species of grasshoppers typically have thermal preferences around 33 °C but can bask periodically to achieve body temperatures between 10 and 15 °C above the ambient temperature, resulting in body temperatures of around 38–40 °C, while *Beauveria* and *Metarhizium* generally have an upper temperature limit for growth of 34–35 °C. (Pepper and Hastings 1952, and Carruthers et al. 1992). The elevated body temperatures also increase metabolism and can speed up development. This accelerated development rate allows them to occupy habitats further north and higher in altitude than they would be able to if they maintained body temperatures close to air temperature (Carruthers et al. 1992). Grasshoppers' natural preference for elevated temperatures is often successful for managing infections, but individuals are capable of elevating their body temperature even higher when faced with infection by pathogens. These higher temperatures severely limit pathogen growth and in some cases are lethal to the pathogens. In strains of *Metarhizium* and *Beauveria*, fever is not lethal to the pathogen. In these situations, fever only serves to severely limit the growth and proliferation of the pathogen within the grasshopper, allowing the host to reproduce and maintain normal functions (Keyser et al. 2014). Once the host is removed from febrile conditions, the pathogen can grow and proliferate unhindered, ultimately killing the host (Elliot et al 2002, Ouedraogo et al. 2003).

Increasingly, research is showing that the balance of macronutrients is important for immune function in insects and that this should be considered in how biopesticides are used for population management (Ponton et al. 2011a, Ponton et al. 2011b, Deans et al. 2017). One potential way to fully enhance the efficacy of biopesticides, such as these fungi, is to use grasshoppers' nutritional physiology against them. For example, Australian plague locusts (*Chortoicetes terminifera* (Walker, 1870)) infected with *M. acridum* increased carbohydrate consumption and decreased protein consumption (Graham et al 2014). Graham et al. (2014) hypothesized that the carbohydrate-biased diets were starving the pathogen of vital sources of protein for growth, reproduction, and production of toxins, and preventing mass colonization in the grasshopper's hemolymph. Corroborating this hypothesis, they found that locusts that ate higher protein diets were more susceptible to *Metarhizium.* However, the effects of diet on biopesticide susceptibility have yet to be tested on other grasshopper species, so it is unknown if this is a broad phenomenon.

The migratory grasshopper, *Melanoplus sanguinipes* (Fabricius), is the most destructive rangeland grasshopper in the United States and is frequently the focus of management efforts because it can cause incredible amounts of damage to crops and rangelands (Pfadt, 2002; Murray, 2016). Because of its destructive nature and economic impact, *M. sanguinipes* is often the subject of many research efforts, however there is minimal research into the synergistic effects of biological control methods and nutritional physiology (Pickford and Mukerji, 1974; Hewitt, 1977; Hewitt and Onsager, 1983; and Pfadt, 2002).

Here, we studied the main and interactive effects of two of the primary environmental factors that affect grasshopper immune responses – temperature, and nutrition – on grasshopper susceptibility to *Metarhizium robertsii* (J.F. Bisch., Rehner & Humber, 2009) strain DWR2009. *Metarhizium robertsii* is moderately pathogenic for Acrididae but, having its origin in the U.S., does not have the same regulatory restrictions as *M. acridium*, which is considered a regulated non-indigenous species with considerable restrictions on laboratory use in the U.S. We hypothesized (1) that migratory grasshoppers (*M. sanguinipes*) actively regulate their nutrient intake to combat *M. robertsii inoculation*, (2) that the elevated temperature treatment groups would have higher rates of survival under *M. robertsii* inoculation, and (3) that eating a prescribed diet that is carbohydrate-biased would lead to higher survival and increase mass with a *M. robertsii* inoculation. Through a series of experiments, we tested if grasshoppers shifted their nutrient intake targets with and without *M. robertsii* inoculation, and with and without elevated thermal treatments, as well as the effects of these treatments on host

mass gain and survival. We then tested the effects of *M. robertsii* on grasshoppers given prescribed (no choice) diets. There has been some research done on the effects of behavioral fever on total consumption (Hajek and St. Leger 1994, and De Faria et al. 1999), but, to our knowledge, this is the first study to test the interactive effects of nutrient balance and elevated temperature on pest susceptibility to biopesticides.

2. Methods

2.1. Host, pathogen, and diets

2.1.1. The migratory grasshopper

The grasshoppers used in this experiment came from the Arizona State University *M. sanguinipes* lab colony. This colony was started from eggs from a USDA-ARS lab colony (Sidney, MT, USA). This colony dates back to 1970 and has had genetic stock added to the colony from several wild non-diapausing populations (Zembrzuski et al. 2021). The colony was established at Arizona State University (ASU, Phoenix, AZ, USA) in 2017, is supported by the USDA-APHIS-Plant Protection and Quarantine (PPQ)-Science & Technology (S&T)-IMMDL (Phoenix Station, Phoenix, AZ, USA) (hereafter referred to as the USDA facilities), and is currently reared on organic romaine lettuce, wheat grass, and wheat bran. The colony is kept at 32° C during the day and 25 °C at night, and the humidity fluctuates between 20 and 50 % RH with a 14 h:10 h light/dark cycle. All diet experiments were carried out at the USDA facilities using grasshoppers from the ASU colony.

2.1.2. Fungal pathogen

For our research we chose to use a species of *Metarhizium* native to the U.S., *Metarhizium robertsii. Metarhizium robertsii* (strain DWR2009) was isolated from soil in grasshopper breeding grounds within the U.S. This species has shown promise for biological control because of its virulence, shelf life, and stability; however, it is a generalist pathogen capable of infecting other taxa besides grasshoppers (Wang et al. 2016). The *M. robertsii* used in this experiment was supplied as a dry conidial powder produced using biphasic solid substrate fermentation by USDA ARS Sidney, MT. Viability of the conidia was $>$ 60% for both experiments on germination tests conducted $<$6 months prior to experiments, as recommended by Stefan Jaronski. Conidia were stored at −25 °C for several months at the USDA facilities.

2.1.3. Artificial diets

Diets were made based on Dadd (1961) and modified by Simpson and Abisgold (1985). All diets were isocaloric, containing 42% macronutrients (protein + carbohydrates). The protein was a 3:1:1 mix of casein, peptone, and albumen (egg whites); the digestible carbohydrate (hereafter, carbohydrates) was a 1:1 mix of sucrose and dextrin. All diets contained similar amounts of Wesson's salt (2.4%), cholesterol (0.5%), linoleic acid (0.5%), ascorbic acid (0.3%) and vitamin mix (0.2%). The remainder of the diet was made up of cellulose. We made different diets for the experiments by varying the percentage of protein and carbohydrate, by dry mass: 7p:35c, 14p:28c, 21p:21c, 28p:14c, and 35p:7c.

2.2. Experiments

2.2.1. Nutrient selection (choice diets) under different temperatures and Metarhizium inoculation

We used a 2 × 2 factorial design where grasshoppers were either inoculated with *Metarhizium* or not and given access to a heating pad or not. We collected 160 adult grasshoppers who had recently molted (within 1–5 days) from colony cages, weighed them and randomly distributed equal numbers of males and females to each group (40 in each treatment group). We then inoculated grasshoppers in the *Metarhizium* groups with a topical application of 1 μl of 5x109 spores/ml (5x106 conidia per grasshopper) suspended in vegetable oil (Kroger brand 100% pure vegetable oil). The mixture was applied directly to the base of the hind coxa of each grasshopper using a Hamilton 50 μl Gastight Syringe Model 1705 TLL, PTFE Luer Lock syringe with blunt-tips and a 1 μl repeater. Grasshoppers in the *Metarhizium*-control groups were treated in the same fashion as the *Metarhizium* group, however instead of applying a fungal pathogen, grasshoppers were touched on their hind coxa with a clean, empty blunt-tipped syringe. No oil carrier control was used as earlier studies showed no impact of oil application on mortality (Bateman et al. 1993). After inoculation, grasshoppers were housed individually in hard plastic cages with air holes (19 cm × 13.5 cm × 9.5 cm), perches, a water tube, and diets.

The experiment room was held at 32.1° C +/− 1.9° C (mean +/− SD), which was the rearing temperature of the grasshopper colony. We placed the cages of grasshoppers in the higher temperature (febrile) groups partially on three 1.2 m by 0.5 m Vivosun heating mats with digital thermostats. Temperatures were recorded from several cages for the duration of the experiment using temperature data loggers. The febrile treatment group average temperature for the heating pad side of the cage was 41.9° C +/− 1.9° C while the average temperature for the other side of the cage without a heating pad was 36.5° C +/− 1.6° C. We selected 42° C as the high temperature because prolonged exposure to temperatures above 35° C stops growth in many entomopathogenic fungi, while even higher temperatures of 41–44° C delays the resumption of normal growth in several *Metarhizium* species (Jaronski 2010). For the lower temperature groups (non-febrile), cages were not placed on heating pads and had an average internal cage temperature of 35.7° C +/− 2.4° C.

We measured the self-selected p:c ratios (termed intake targets, IT) of the grasshoppers in the above treatment groups by giving them access to two nutritionally complementary artificial powdered diets served in two identical small plastic dishes for each locustone low p:c and one high p:c. To determine if grasshoppers in each treatment group were actively selecting for a specific p:c ratio and not simply eating randomly from between the two dishes, we split each of the four main treatment groups into two sub-groups, with each group receiving a different diet pairing. Both diet pairs included two complementary isocaloric diets (Pair A: 7p:35c and 28p:14c; Pair B: 7p:35c and 35p:7c). The initial weight of each diet dish was recorded and then diets were placed in cages with the grasshoppers. If grasshoppers were alive on day six, diet dishes were replaced with fresh diet dishes. We dried the dishes from days 0–6 for 36 h at 60 °C, removed any frass, then weighed the dish with the remaining diet to calculate consumption from the two diet dishes for the first six days. For grasshoppers that died between days four to six, we calculated their consumption up to the day before they died. The diet dishes of any grasshoppers that died prior to day four were removed from consumption analyses. We calculated consumption as a daily rate of mass specific nutrients eaten.

Throughout the experiment, grasshoppers were checked daily for mortality. Dead grasshoppers were removed, weighed, and placed into labeled Petri dishes. Petri dishes were placed into plastic containers with wet paper towels and placed in a fungal growth chamber set to 29°C with relative humidity reaching between 98% and 100%. Dead grasshoppers were checked daily for characteristic outgrowth and sporulation. At the conclusion of the experiment on day 12, any surviving grasshoppers were weighed, euthanized by oxygen deprivation, and placed in a fungal growth chamber.

2.2.2. Survival under prescribed diets and Metarhizium inoculation

For this experiment, we transferred egg cups from the ASU colony to the USDA facilities. Once hatched, grasshoppers were reared on 12 h:12 h day/night cycle, and approximately 32 °C day/night temp. Grasshopper nymphs were fed wheat grass seedlings (grown at ASU) and wheat bran. We distributed young adult grasshoppers into three large cages and continued their diet of wheat grass and wheat bran until we had enough grasshoppers for our treatment group sizes with extras in case of mortality. In total, we started with 168 males and 202 females split evenly across the three cages, and all grasshoppers were within 5

days since adult eclosion. At this time grasshoppers were moved to the experiment room where the average temperature was 34.1℃ +/− 2.2℃ and switched to feeding on one of three artificial diets (7p:35c, 21p:21c, or 35p:7c), with water provided ad libitum in cotton stoppered vials, for 3 days. Grasshoppers were then weighed and assigned to an individual vented plastic cage (19 cm × 13.5 cm × 9.5 cm), given their same artificial diet, a water tube, and a perch, then either inoculated with *M. robertsii* or assigned as an untreated control.

For the *M. robertsii* inoculation, grasshoppers were handled in the same manner as the nutritional choice experiments, with the exception that *Metarhizium*-treated individuals received a lower dose, a 1 μl topical application of $1x10^9$ spores/ml. We used a lower dose for this experiment as we were not using febrile temperatures and expected the grasshoppers to have lower survivability without the thermal factor. There were six treatment groups: 1) *Metarhizium*-treated with diet 7p:35c, 2) *Metarhizium*-treated with diet 21p:21c, 3) *Metarhizium*-treated with diet 35p:7c, 4) No *Metarhizium* with diet 7p:35c, 5) No *Metarhizium* with diet 21p:21c, and 6) No *Metarhizium* with diet 35p:7c. Each *Metarhizium* treatment group had 30 grasshoppers (15 male and 15 female) while each treatment group without *Metarhizium* had 20 grasshoppers (10 male and 10 female) – 150 grasshoppers total. The inoculated and control groups had different numbers due to space and supply limitations. During this time, grasshoppers were kept at the same day/night light and temperature cycle as they were in their communal cages. Diets were changed every 3 days, and water was added as needed for 18 days. The grasshoppers were checked daily for mortality. Dead grasshoppers were removed, weighed, and placed into labeled Petri dishes and kept at saturated humidity to elicit characteristic fungal outgrowth and sporulation. At the end of 18 days, any surviving grasshoppers were euthanized, weighed, and incubated at high humidity. Cadavers were kept at high humidity for up to 2 weeks after the experiment, then photographed and visually inspected for *Metarhizium* growth. Survival data was collected and used to calculate survival curves and median survival times (MST). We calculated specific growth rate (SGR) for uninoculated grasshoppers that survived to the end of 18 days using the following formula μ= (ln (M1/M2))/dt, where M1 is the initial mass of the grasshopper, M2 is the final mass of the grasshopper, and dt is the days between weight measurements.

2.3. Statistical analyses

We tested all data for assumptions of normality and homoscedasticity implicit in parametric tests. To test if grasshoppers were consuming randomly from diet dishes or not, we used a Mann Whitney Wilcoxon Test. To test specifically for a shift in IT, we ran an ANOVA on the ratios of p:c consumed using the *anova* function with type III sum of squares from the *car* package (v3.0–12) in R. SGR data was analyzed with a 1-way and 2-way ANOVA. Grasshopper survival was analyzed with Kaplan Meier survivorship analysis using the *survfit* function from the R *survivor* package (v3.2–11) with multiple comparisons performed using the *pairwise_survdiff* function from *Survminer* (v0.4.9) with the Benjamini–Hochberg method implemented for controlling false discovery. Postmortem *Metarhizium* growth presence or absence data was analyzed using a Fisher's test of Independence. We performed all analyses using R 3.5.1 (2018).

3. Results

3.1. Nutrient selection (choice diets) and survival under febrile conditions and Metarhizium inoculation

In all treatment groups, grasshoppers ate non-randomly from the two diet dishes (Mann Whitney Wilcoxon Tests; Table 1) and selected a carbohydrate-biased IT of approximately 1p:2c (Fig. 1A). However, an ANCOVA showed that inoculated grasshoppers without heat ate substantially less overall than the other treatment groups (Fig. 1B; Table 2).

Table 1
Mann Whitney Wilcoxon Test for non-normal data to determine, for all treatments, that consumption from the two diet choices in the first experiment was different than would be expected by random (eating equally from both dishes). The V value is the measure of similarity between values being compared. Significant P values (<0.05) indicate that grasshoppers were not eating randomly and were instead selecting for a given mix of nutrients. Pair A: 7p:35c and 28p:14c; Pair B: 7p:35c and 35p:7c, percent protein:carbohydrate by dry mass.

Treatment	V	P value
Met + Heat Diet pair A	208	5.72E-06
Met + Heat Diet pair B	210	1.91E-06
Met + No Heat Diet pair A	183	0.002325
Met + No Heat Diet pair B	206	1.34E-05
No Met + Heat Diet pair A	210	9.56E-05
No Met + Heat Diet pair B	210	1.91E-06
No Met + No Heat Diet pair A	210	1.91E-06
No Met + No Heat Diet pair B	210	9.56E-05

To test specifically for a shift in IT, we ran an ANOVA on the ratios of p:c consumed, which showed no significant main or interactive effects of heat or *Metarhizium* inoculation (Table 3), indicating that grasshoppers did not shift their IT in response to those treatments. While analyzing ratios can be problematic, we were able to address several concerns raised by Raubenheimer (1995): our data were constrained between 0.2 and 5p:c based on the diets offered, there were no negative values, and we log-transformed ratios prior to analysis. Another analysis, such as this ANOVA on p:c ratios, is important to include to test for relative consumption of p and c when there is a substantial difference in total macronutrients consumed between different treatment groups; solely using results from the MANCOVA approach standard in Geometric Framework studies (e.g., Chambers et al. 1995) would lead to the erroneous conclusion that inoculated grasshoppers without heat selected a different p:c ratio when all groups fall on the 1p:2c line (Fig. 1A). Survival curve analyses show that there was significant difference in survival probability among all treatment groups ($P < 0.0001$), pairwise comparisons using log-rank tests showed the *Metarhizium* no-heat treatment group having a lower survival than all other treatment groups (Fig. 2). Median survival times (MST) are reported in Table 4.

3.2. Survival under prescribed diets and Metarhizium inoculation

For the no choice experiments, we determined specific growth rate for uninoculated grasshoppers that survived the duration of the experiment and found there was no significant difference in SGR between diet treatments (ANOVA: $F(2,17) = 1.40$, $P = 0.28$). We also determined there was no effect of sex on SGR (ANOVA: $F(1,16) = 1.04$, $P = 0.32$). Mean (+/− SE) SGR for surviving grasshoppers from uninoculated treatment groups was −0.025 +/− 0.011 (7p:35c), −0.014 +/− 0.010 (21p:21c), and −0.004 +/− 0.003 (35p:67c). We did not test growth rate for the *Metarhizium*-treated groups because survival dropped off quickly. We then used a Kaplan Meier Survivorship analysis on all treatment groups that showed there was a significant difference in survival through time among treatment groups ($P < 0.0001$) (Fig. 3). Median survival times (MST) are reported in Table 5 for all treatments. There were no significant differences in survival among diet groups of uninoculated grasshoppers ($P = 0.630$) (survival curves shown in Fig. 3); however, there were significant differences in survival through time among diet groups in *Metarhizium* treated grasshoppers ($P = 0.0022$) (Shown in Fig. 3). Additionally, pairwise comparisons using log-rank tests showed that inoculated individuals fed the 21p:21c diet died sooner than inoculated individuals eating either the 7p:35c or 35p:7c diets (Fig. 3).

The final analysis we conducted was a Fisher's test of independence that showed there was a significant difference in *Metarhizium* sporulation post-mortem among treatment groups ($P < 0.0001$) (Fig. 4). Inoculated grasshoppers fed a 21p:21c diet had a higher occurrence of post-

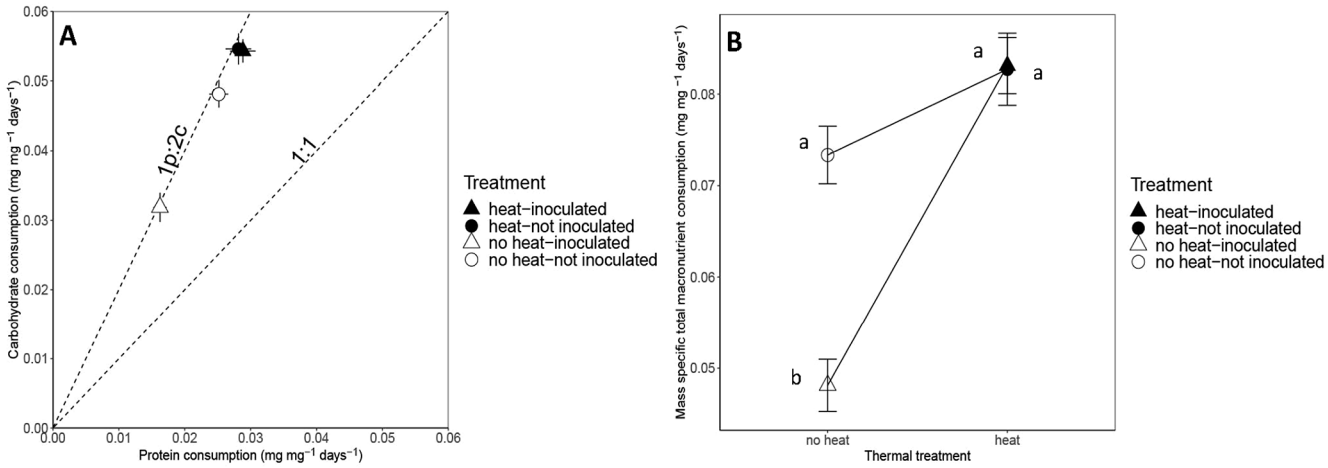

Fig. 1. Panel A: Carbohydrate to protein intake ratios all fell on an approximately 1p:2c dietary rail. Points represent means +/− SEM. Panel B: In the intake target experiment, there was a significant two-way interaction between heat and *Metarhizium* on total macronutrients consumed while controlling for start body mass (ANCOVA $F(1, 139) = 32.5$, $P < 0.001$). Lowercase letters indicate post hoc difference from Tukey HSD analyses on mass specific feeding rates. All consumption data are daily rates for up to the first six days of the experiment.

Table 2
Analysis of Covariance (ANCOVA) testing the effect of inoculation and temperature on total macronutrient consumption. "Ges" or generalized eta squared is the effect sized used in the ANCOVA.

Effect	DFn	DFd	F	p p < 0.05	ges
Grasshopper Start mg	1	134	0.000482	9.83E-01	3.60E-06
Heat	1	134	77.347	6.19E-15	* 3.66e-01
Metarhizium	1	134	28.656	3.64E-07	* 1.76e-01
Sex	1	134	0.348	5.56E-01	3.00E-03
Heat × *Metarhizium*	1	134	31.919	9.25E-08	* 1.92e-01
Heat × Sex	1	134	2.515	1.15E-01	1.80E-02
Metarhizium × Sex	1	134	0.04	8.42E-01	2.98E-04
Heat × *Metarhizium* × Sex	1	134	2.484	1.17E-01	1.80E-02

Table 3
Analysis of variance (ANOVA) statistics testing the effect of heat, *Metarhizium*, and sex on the ratios of protein and carbohydrate consumed.

	Sum Sq	Df	F value	Pr (>F)
Intercept	1.0773	1	74.6216	$1.43e^{-14}$
Heat	0.0002	1	0.0160	0.8997
Metarhizium	0.0015	1	0.1045	0.7470
Sex	0.0044	1	0.3067	0.5806
Heat × *Metarhizium*	0.0012	1	0.0856	0.7703
Heat × Sex	0.0003	1	0.0220	0.8824
***Metarhizium* × Sex**	0.0017	1	0.1184	0.7313
Heat × *Metarhizium* × Sex	0.0040	1	0.2771	0.5995
Residuals	1.9489	135		

mortem *Metarhizium* growth relative to all other treatment groups (Fig. 4). Furthermore, using a pairwise nominal independence post hoc test we found the small proportions of *Metarhizium* growth found in the inoculated groups fed the 7p:35c or 35p:7c diets was not statistically different from zero, the amount of *Metarhizium* growth found in the uninoculated treatment groups.

4. Discussion

Biopesticides can be viable alternatives to traditional pesticides due to their decreased environmental and non-target impacts, however, logistical challenges remain, in part due to a particular pest's capacity to resist/overcome the effects of a given biopesticide. Our results revealed that thermal environment and dietary macronutrients can both affect the susceptibility of the grasshopper *Melanoplus sanguinipes* to *Metarhizium robertsii* inoculations. Elevated temperature opportunities were more powerful as a defense than diet and *M. sanguinipes* specimens did not regulate p:c consumption in response to inoculation. However, an unexpected increase in the survival of the grasshoppers fed carbohydrate- or protein-biased diets relative to individuals fed balanced diets suggests, depending on the diet bias, that at least this species gains resistance benefits through different mechanistic pathways.

Access to elevated temperature in the *M. sanguinipes* specimens was extremely effective against *Metarhizium*. Febrile opportunity (access to 42 °C heating mats) rescued grasshoppers from high-dose fungal inoculations who survived at similar rates to uninoculated grasshoppers, while most inoculated grasshoppers without febrile conditions died within 5–6 days (Fig. 2). These results corroborate other studies that showed the impact of higher temperatures on survival for a multitude of grasshopper and locust species during fungal, bacterial, and microsporidian pathogen challenges, such as *Beauveria, Metarhizium, Nosema*, and *Serratia* (Boorstein and Ewald 1987, Inglis et al. 1996, Blanford and Thomas 2000, Elliot et al. 2002, Bundey et al. 2003, Ouedraogo et al. 2003, Clancy et al. 2018, and Sangbaramou et al. 2018). For example, the migratory locust, *Locusta migratoria* (Linnaeus, 1758), has been observed using behavioral fever to fight both *B. bassiana* and *M. acridum* infections. In both cases, as little as a couple hours thermoregulating to febrile conditions was enough to increase in survival while infected. Longer exposure elicited higher survival rates with 4 h of thermoregulation increasing survival under *B. bassiana* by 43.34%, and by 85% under *M. acridum* infections (Ouedraogo et al. 2003, and Sangbaramou et al. 2018). The increased body temperatures due to behavioral fever in *L. migratoria* were also accompanied by increased occurrences of hemocytes and phagocytic activity, which serve to fight off infection (Sangbaramou et al. 2018). The desert locust, *Schistocerca gregaria* Forsskål, 1775, infected with *M. acridum* actively increased body temperature through basking and posturing in optimal conditions for fungal resistance in response to infection, in some being able to achieve body temperatures consistent with fever when unrestricted (Elliot et al. 2002; Bundey et al. 2003). Behavioral fever, in most cases, did not rescue *S. gregaria* completely, but increased survival to about 21 days, leaving enough time for infected grasshoppers to mate and produce viable offspring (Elliot et al. 2002). Further studies with this species also suggest that not only are the locusts actively thermoregulating to febrile levels, but the dose impacts the magnitude of the febrile response,

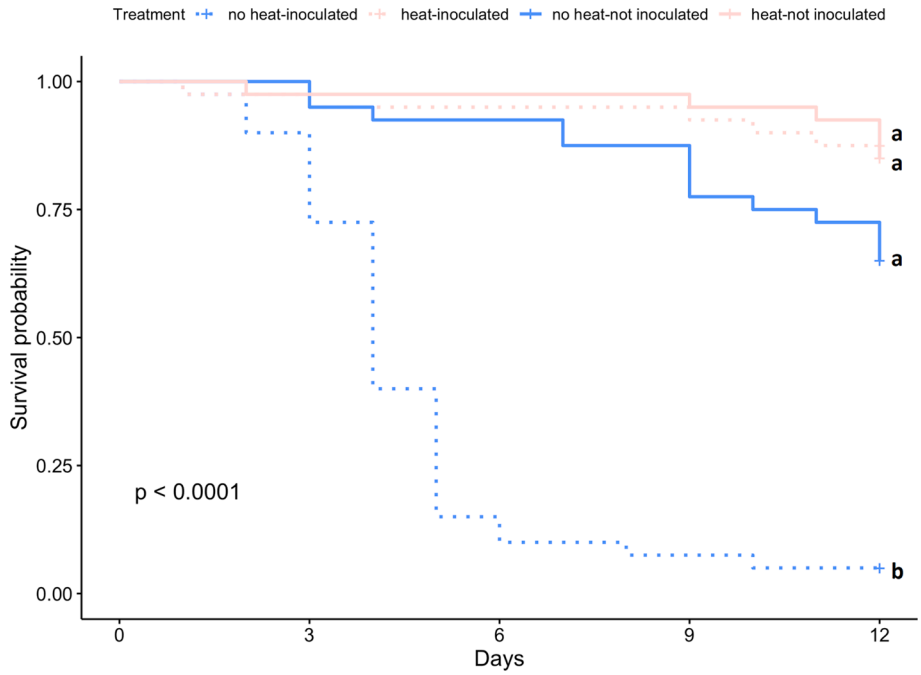

Fig. 2. Kaplan Meier Survival Curve from the elevated temperature and diet choice experiment. Letters on right side represent significance and were determined using pairwise comparisons using log-rank tests. Inoculated grasshoppers with no heat died the quickest; there were no differences among the other three treatment groups.

Table 4
Median Survival Times (MST) and their 95% confidence levels from the elevated temperature and diet choice experiment.

Treatment	MST	95% lower confidence level	95% upper confidence level
No Heat- Met	4	4	5
Heat- Met	>12	NA	NA
No Heat- No Met	>12	NA	NA
Heat- No Met	>12	NA	NA

meaning that locusts infected with higher doses of *Metarhizium* elevated their body temperature higher than those infected with lower doses (Clancy et al. 2018).

While much of the research into behavioral fever has focused on locust species that are common targets for biological control, there is also significant research into behavioral fever observed in *M. sanguinipes*. Most of this research focuses on behavioral fever under *Beauveria* and *Nosema* challenges. For example, when infected with *Nosema acridophagus* or *B. bassiana, M. sanguinipes* selected hotter temperatures and actively basked to reach higher temperatures (Boorstein and Ewald 1987, and Inglis et al. 1996). *Melanoplus sanguinipes* utilizing behavioral fever to fight *N. acridophagus* increased survival and growth rates compared to individuals who were restricted from thermoregulating, however, after 10 days of febrile conditions the impact to growth rate diminished (Boorstein and Ewald 1987). Behavioral fever was also highly effective at rescuing *M. sanguinipes* infected with *B. bassiana*, as exposure to temperatures between 35 and 40 ° C for at least 6 h decreased disease by 98% (Inglis et al. 1996). Our research now further expands this growing body of literature by showing that febrile conditions increased survival in *M. sanguinipes* inoculated with high (1 μl of $5x10^9$ spores/ml) dosages of *M. robertsii.*

The exact mechanisms of behavioral fever induction are still being studied, but current febrile conditions can be artificially induced in some species of Orthoptera, such as *Gryllus texensis* (Cade and Otte 2000) and *Schistocerca gregaria,* by injecting eicosanoids, like prostaglandin, or eicosanoid precursors into the insect's hemolymph (Bundey et al. 2003, and Stahlschmidt and Adamo, 2013). Febrile responses can also be prevented by inhibiting eicosanoid synthesis, suggesting pathogens trigger the synthesis of eicosanoids, which, in turn, initiates a febrile response (Bundey et al. 2003, and Stahlschmidt and Adamo, 2013). However, there are some pathogens that elicit febrile responses but evade detection by the insect, like *M. acridum*, which is capable of evading immune detection once hyphal bodies reach the hemolymph (Wang and St. Leger 2006). Once in the hemolymph, rapid expression of the *Mcl1* gene is triggered, which produces a collagenous coat that blocks β glucan receptors that are essential for the pathogen-associated molecular pattern (PAMPs) recognition. Even though *Metarhizium* infections can evade detection they still induce febrile responses in grasshoppers, so potentially the signal that induces the febrile response occurs prior to hemolymph colonization, during cuticle penetration or earlier (Wang and St. Leger 2006, and Clancy et al. 2018).

In addition to fever, the ratio of macronutrients is important for immune function and may be important to consider when biopesticides are used for population management (Srygley and Lorch, 2011, Ponton et al. 2011a, Ponton et al. 2011b, Srygley 2016, Deans et al. 2017, Srygley 2017, Tessnow et al. 2017, Srygley and Jaronski 2018). For example, research using prescribed diets with *C. terminifera* locusts infected with the biopesticide *Metarhizium* showed that measures of immune response (lysosome-like antimicrobial activity and hemocyte density) were lower on the low protein high carbohydrate prescribed diet compared to the high protein low carbohydrate prescribed diet (Graham et al. 2014). Studies on Mormon crickets (Anabrus simplex Haldeman, 1852) looking at the importance of diet on immune function show that both carbohydrates and protein are important for different aspects of immune responses like phenoloxidase (PO) activity and lysozyme-like activity (Srygley and Lorch, 2011). This research showed a band of Mormon crickets from Nevada, USA (with a carbohydrate preference) had reduced movement and a greater total PO activity and greater encapsulation response to the introduction of a foreign object when fed a carbohydrate rich diet as opposed to a protein rich diet. There was no difference in spontaneous PO activity, which is an important aspect of fighting a *B. bassiana* infection (Srygley and

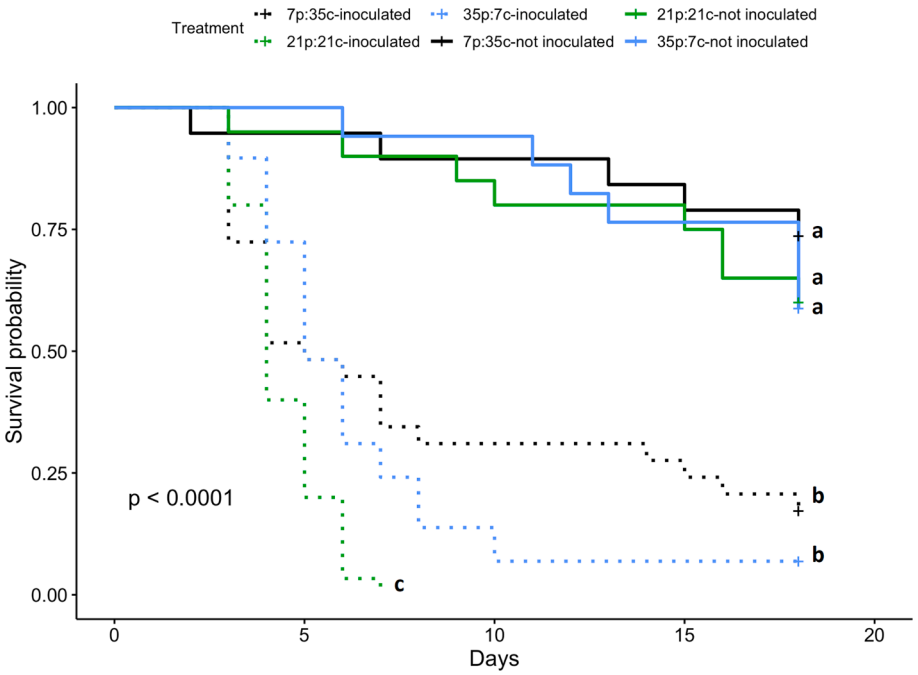

Fig. 3. Kaplan Meier Survival Curve analysis for all treatment groups from the prescribed diet experiment. Letters on right side represent significance and were determined using pairwise comparisons using log-rank tests and false discovery rate controlled with Benjamini-Hochberg method. Inoculated grasshoppers fed the balanced diet died sooner than those fed the carbohydrate or protein biased diets.

Table 5
Median Survival Times (MST) and their 95% confidence levels from the prescribed diet experiment.

Treatment	MST	95% lower confidence level	95% upper confidence level
Met + 7p:35c	5	4	14
Met + 21p:21c	4	4	5
Met + 35p:76c	5	5	7
No Met + 7p:35c	>18	NA	NA
No Met + 21p:21c	>18	NA	NA
No Met + 35p:76c	>18	NA	NA

Jaronski, 2011; and Srygley and Lorch, 2011). Srygley and Lorch (2011) also looked at a band of Mormon crickets from Utah that was protein limited and found that this band preferred a protein rich diet, and ultimately had higher spontaneous PO activity when feeding on protein rich diets. Studies on lab reared Mormon crickets looking at carbohydrate and protein intake have shown that in the absence of macronutrient limitations both nymphs and adults prefer diets that maximize macronutrients and tend to balance carbohydrates and proteins (Srygley 2017). Another study on laboratory reared Mormon crickets, without macronutrient limitations, show that specimens had higher phenoloxidase titers, better encapsulation, and higher survival when faced with *B. bassiana* infection when consuming high protein diets as opposed to low protein diets (Srygley and Jaronski 2018).

There are a multitude of proteins involved in the signal pathways and immune responses of insects (Strand 2008) and high protein diets may support these. In both *S. gregaria* and *L. migratoria*, host hemolymph protein levels fall during *Metarhizium* infections, which could coincide with the pathogen consuming the protein from the hemolymph or could coincide with increased immune activity drawing on protein stores in the host (Gillespie et al. 2000, Mullen and Goldsworthy 2006). In some cases, like the infection of the beet armyworm (*Spodoptera exigua* (Hübner, 1808)) with *Beauveria bassiana,* protein synthesis remains normal during the vegetative development of the pathogen in the haemocoel but is inhibited once the fungus mycelium invades host tissue. At this time, the fungal hyphal bodies began producing toxic metabolites and enzymes that dissolve the host tissue (Mazet and Boucias 1996). While protein consumption is vital for immune function, it might not be what helps some insects evade *Metarhizium* infections. A similar study from Graham et al. (2014), found that *C. terminifera* locusts that selected more carbohydrate-biased diets were less susceptible to the fungal pathogen *Metarhizium*. The authors hypothesized that the carbohydrate-biased diets were selected by grasshoppers and that these diets improved survival by starving the pathogen of vital sources of protein for growth and reproduction, thereby preventing mass colonization in the grasshopper's hemolymph.

Our experiments add an additional level of perspective to the complexities of insect nutritional immunology. In the prescribed-diet experiments, *Metarhizium*-inoculated grasshoppers, eating the carbohydrate-biased (7p:35c) and protein-biased (35p:7c) diets, survived longer than those fed the balanced (21p:21c) diet (Fig. 3). In addition, post-mortem *Metarhizium* sporulation was greatest on grasshoppers fed the balanced (21p:21c) diet (Fig. 4). We propose a min–max hypothesis to explain this outcome, whereby one factor needs to be minimized and the other maximized for there to be a significant effect of that factor: in this case, carbohydrate and protein intake. Potentially, eating a protein-biased diet supported an effective immune response, whereas eating a carbohydrate-biased diet starved the pathogen of protein and/or supported an effective immune response via different pathways. Eating the balanced 21p:21c diet either did not support the host to mount an effective immune response, provided a nutritionally optimal environment for pathogen growth, or both. Interestingly, even though a large proportion of inoculated grasshoppers eating protein- and carbohydrate-biased diets died, eating these diets precluded substantial fungal growth even in the cadavers. It is important to note that the temperature these experiments were conducted under (34.1℃ +/− 2.2℃), is known to inhibit fungal growth, however we did observe fungal growth on a proportion of cadavers from each Metarhizium treatment group. The small proportions of *Metarhizium* growth found in the high carbohydrate and high protein treatment groups was not statistically different from zero, the amount of *Metarhizium* growth found in

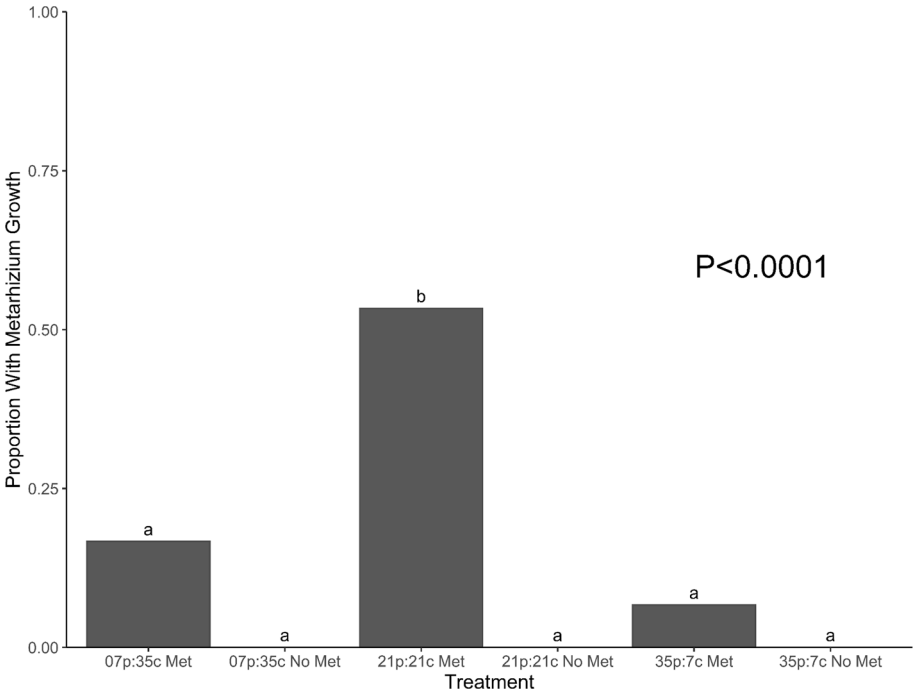

Fig. 4. *Metarhizium* growth post-mortem, letters on top of the bars represent post hoc significance. Inoculated grasshoppers fed the balanced diet (21p:21c Met) had a higher proportion of fungal growth than any of the other treatment groups.

the cadavers of uninoculated treatment groups. These results suggest that populations encountering and eating highly skewed protein biased or carbohydrate diets would be much less likely to spread the biopesticide *Metarhizium* amongst other grasshoppers, thereby decreasing overall effectiveness of the biopesticide on grasshopper population suppression. Additionally, even though diet did not have a large impact on rescuing survival and if dead grasshoppers are not exhibiting fungal growth after they die, the impacts the fungus had on the population might still be limiting in other ways.

Despite the potential advantage of shifting macronutrient balance in response to inoculation, grasshoppers selected a consistent 1p:2c IT, regardless of *Metarhizium* or heat treatment (Fig. 1), indicating that they were not selecting their food to fight the pathogen. This may be because shifting macronutrient balance was not as effective at rescuing inoculated insects as elevated temperature was, which grasshoppers may be able to commonly induce in field settings. We used a lower *Metarhizium* dose for the prescribed diet experiment than the nutrient selection and thermal effects experiment Yet, even grasshoppers on the prescribed diets that best defended them against *Metarhizium* experienced high mortality rates relative to uninoculated control grasshoppers fed the same diets (Fig. 3). In contrast, febrile temperature grasshoppers inoculated with a high *Metarhizium* dose-maintained survival rates indistinguishable from uninoculated control groups (Fig. 2). Non-mutually exclusive explanations for a lack in IT shift could be that the 1p:2C ratio was carbohydrate-biased enough to confer the beneficial protection from *Metarhizium* and/or that this ratio optimized other life history parameters that prioritized overusing diet to suppress *Metarhizium*. Additionally, these experiments were performed on adult grasshoppers. Previous IT experiments on this colony indicated that final instar nymphs have a baseline IT close to 1p:1c (Zembrzuski et al., 2021). Research shows that some adult grasshoppers, after an initial post-molt period of growth, tend to maintain carbohydrate biased ITs for general maintenance, suggesting that the colonies baseline carb biased IT was normal for their age (Chyb and Simpson, 1990). If this experiment were repeated on juvenile grasshoppers, we might see the shift to a higher carb diet that we had expected to see in response to *Metarhizium* inoculation in this study, as nymphs select balanced 1p:1c in normal lab conditions (Graham et al. 2014, and Zembrzuski et al., 2021). Research into insects shifting macronutrient balance in response to infections adds to the growing body of literature showing that insects are capable of making nutritional choices based on macronutrient content as well as plant secondary metabolites to combat pathogens (Lee et al. 2006, Povey et al. 2009, Singer et al. 2009, Cotter et al. 2011, Srygley and Lorch, 2011, Abbott 2014, Srygley 2016, Srygley 2017, Srygley and Jaronski 2018 de Roode and Hunter 2019).

Biopesticides are often tricky to use efficiently in the field. One reason is behavioral fever, which leaves a very narrow window of temperatures for fungal pathogens to work effectively at managing insect pests. Our research supports the notion that behavioral fever could be a big concern in using a fungus like *M. robertsii* for management of *M. sanguinipes*. However, the added nutritional studies provide us with a few tools that might help make *Metarhizium* applications more efficacious. Understanding the nutritional physiology and the nutritional landscape of *M. sanguinipes* could help with biopesticide treatments (Zembrzuski et al. 2021). It is important to note that experiments conducted on laboratory colonies, may not translate directly to field studies or natural populations due to years of artificial selection occurring on laboratory colonies, and therefore studies should be replicated on natural populations and in field settings to verify the relevancy of lab-based studies. Given our finding that balanced diets provided the most opportunity for *Metarhizium* inoculation leading to increased specimen mortality and subsequent sporulation, use of this biopesticide could be targeted in areas where the nutritional landscape makes grasshoppers most vulnerable to infection. To support development of sustainable management options, future research should study the combined effects of behavioral fever and prescribed diets under different biopesticide challenges, as well as behavioral studies, using more pest grasshopper species, and a wider range of macronutrient ratios, to fully understand the relationship between nutritional physiology of grasshoppers and biopesticide efficacy.

CRediT authorship contribution statement

Deanna Zembrzuski: Supervision, Visualization, Methodology,

Writing – review & editing, Writing – original draft, Resources, Investigation, Formal analysis, Conceptualization. **Derek A. Woller:** Funding acquisition, Supervision, Resources, Conceptualization, Writing – review & editing. **Stefan Jaronski:** Methodology, Conceptualization, Writing – review & editing, Resources. **Lonnie R. Black:** Writing – review & editing, Resources, Investigation. **K. Chris Reuter:** Writing – review & editing, Resources. **Dustin Grief:** Writing – review & editing, Investigation. **Alonzo Beatty:** Writing – review & editing, Investigation. **Rick Overson:** Visualization, Formal analysis, Methodology, Conceptualization, Writing – review & editing, Writing – original draft, Resources. **Arianne J. Cease:** Funding acquisition, Supervision, Visualization, Methodology, Writing – review & editing, Writing – original draft, Resources, Formal analysis, Conceptualization.

Declaration 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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105268.

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