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Defensive compounds of *Blissus pulchellus* (Hemiptera: Blissidae) as a barrier against infection by entomopathogenic fungi

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Abstract

Defensive secretions produced by certain hemipterans are known to deter natural enemies and play a crucial role in reducing microbial infections. In this study, we investigated the protective mechanisms of the chinch bug *Blissus pulchellus* against entomopathogenic fungi and we explored the relationship between the major volatile compounds produced by *B. pulchellus* and their potential role in enhancing its resilience to disease. Both adults and nymphs exhibited low susceptibility to infection by various strains of *Metarhizium anisopliae* and *Beauveria bassiana*. The close and continuous contact of conidia with antimicrobial substances on the insect's integument significantly inhibited germination rates. Conidia washed from insects after 4 h of contact with their integument exhibited germination rates of less than 20% on culture media. Chemical analyses of body extracts from adults and nymphs revealed both qualitative and quantitative differences in their defensive compound profiles. Our findings suggest that the aldehydes are the primary compounds responsible for fungal inhibition, effectively protecting the insect from infection. Identifying fungal strains capable of overcoming the fungitoxic compounds produced by *B. pulchellus* is crucial for advancing mycopesticide development to manage chinch bug populations in pastures.

KEYWORDS
aldehydes, *Beauveria bassiana*, chinch bug, fungitoxicity, *Metarhizium anisopliae*

INTRODUCTION

The chinch bug *Blissus* spp. (Hemiptera: Blissidae) is an important pest of cereal crops and grasses in many parts of the Americas (Baxendale et al., 1999; Fidelis et al., 2021; Reinert et al., 2011; Spike et al., 1994). Huge populations of chinch bugs infesting and damaging cereal crops in the Midwest plains of the US in mid-to-late 1800's were cited as one of the major causes for farmers to abandon the production of cereals in those areas (Thomas, 1879; Thompson, 1909). Since the 1950's, chinch bugs have also been reported in Brazil, occasionally damaging different grass varieties (Costa, 1945; Reis et al., 1976; Valério et al., 1999). *Blissus antillus* (initially misidentified as *B. leucopterus*) was reported attacking Tanner grass (*Brachiaria arrecta*) and Tangola (hybrid of *B. arrecta* and *B. angola*) in Southeast, Midwest, North and Northeast of Brazil from 1975 to the early 2000's (Valério, Valério et al., 2015). More recently, great numbers of *Blissus pulchellus* were detected in areas of pasture in the Amazon region, causing plant death, pasture degradation and negative impacts on forage production (Fidelis et al., 2021). In the North region of the country, probably favoured by prolonged droughts, insect density reached 8500 chinch bugs/m^2 in the beginning of 2016. Other cattle producing areas in Mato Grosso and Rondônia states are also facing

the same problem, and farmers have been reporting high infestations and substantial damage to their pastures in the last years (Fidelis pers. comm.).

Interestingly, as chinch bugs devastated U.S. wheat fields toward the end of the 19th century, an infectious disease afflicting their populations was concurrently reported (Shimer, 1867; Snow, 1891). Henry Shimer thoroughly wrote in 1867—'*The majority of the chinch bugs yet alive are in the imago state, but they are being rapidly destroyed by the prevailing epidemic disease, more fatal to them than the plague or Asiatic cholera ever was to man, more fatal than any recorded disease among men or animals since time began. Scarcely one in a thousand of the vast hosts of young bugs observed at the middle of June yet remain alive, but plenty of dead ones may be seen everywhere, lying on the ground, covered with the common mould…*' (Shimer, 1867)—referring to the epizootic occurrence of an entomopathogenic fungus, probably an entomophthoralean species. The extensive application of an endemic *Beauveria bassiana* against chinch bugs in that same region of the US a few years later is often cited as one of the earliest attempts to control a pest using an insect pathogen (Lord, 2005), but the real impact of those applications was inconclusive (Billings & Glenn, 1911).

A hundred years after the first attempt to biologically control chinch bug populations in the field, new efforts have been made to better understand the complex fungal pathogen-*Blissus* interactions. Several factors, including abiotic conditions (such as moisture and temperature), microorganism species or strain and the host's developmental stage have been shown to have great influence on fungal performance (Boyle & Cutler, 2012; Krueger et al., 1991; Ramoska, 1984; Samuels et al., 2002; Samuels & Coracini, 2004). More importantly, these studies indicate that disease occurs successfully only when insects are exposed to high concentrations of infective propagules, revealing a possible protective mechanism in adults and nymphs against entomopathogenic fungi.

Defensive secretions from fungus-resilient pentatomids are known to exert a strong inhibitory effect on conidia germination (Borges et al., 1993; Lopes et al., 2015; Silva et al., 2015; Sosa-Gómez et al., 1997). Nevertheless, few studies have investigated the existence of defensive compounds in Blissidae insects and their effect on host invasion by entomopathogenic fungi. In a preliminary study, Boyle and Cutler (2012) reported that cuticular extracts from adults of *Blissus leucopterus* exhibit conidia-inhibiting activity, though the specific components of these extracts were not identified. The secretions produced by the grain chinch bug *Macchiademus diplopterus* include several common hemipteran defensive compounds, such as hexanal, (*E*)-2-hexenal, (*E*)-2-hexenol, (*E*)-2-hexenyl acetate, (*E*)-2-octenal, (*E*)-2-octenol, (*E*)-2-octenyl acetate and tridecane (Okosun, 2012), but their effect on infective fungal propagules has not been determined.

In the present study, we identified the composition of the major defensive compounds of adults and nymphs of *B. pulchellus*, evaluated the effectiveness of *Beauveria* and *Metarhizium* strains applied to both insect stages, and hypothesized, based on the defensive blends, the host defence mechanisms involved in the infection process.

MATERIALS AND METHODS

Insect collection and colony maintenance

Adults and third-instar nymphs of *B. pulchellus* used in all the experiments were obtained from colonies established in May 2023 at Embrapa Genetic Resources and Biotechnology (Federal District, Brazil). Insects were collected from infested pasture areas (*Brachiaria humidicola*) in the state of Mato Grosso, Brazil (15° 14′ 08.38″ S, 15° 11′ 15.95″ W) in the same month (approximately 500 insects).

The colonies were maintained indoors on the same grass, grown in plastic trays (20 × 45 × 10 cm) at 29 ± 2°C, under a set of full-spectrum LED grow light (Quantum Barra PRO, 240 W, red 660 nm + blue 450 nm, Masterplants) positioned 60 cm above the trays, with a 14:10 h light: dark photoperiod.

Trays with plants were replaced every 2 weeks or as needed, and insects were manually transferred to the new grass. Dead insects found in the colony during the first 3 months before the bioassays were kept in wet chambers for 5 days at 26 ± 0.5°C to check the natural incidence of entomopathogenic fungi in the field-collected population.

Susceptibility of *Blissus pulchellus* to different *Metarhizium* and *Beauveria* strains

In the first bioassay, 10 fungal strains from the Invertebrate-Associated Fungal Collection (CFI) maintained at Embrapa Genetic Resources and Biotechnology (Supplementary Table S1) were evaluated against adults on grass in cages. Aerial conidia from all the strains were collected from 15-day-old cultures grown on potato dextrose agar (PDA), suspended in sterile distilled water plus the surfactant, Tween 80 (0.05% v/v), and adjusted to a final concentration of 4×10^7 viable conidia mL^{-1}. Twenty-five adults collected with a brush directly from the colonies were transferred to *B. humidicola* plants (15 cm tall) grown in pots (300 mL) with sterile substrate.

Plants and insects were then sprayed with a 3 mL conidial suspension (ca. 1×10^5 conidia/cm^2) using an airbrush attached to an electric compressor (Elite-175X Oil-less 1/5 HP, PointZero Airbrush, FL, USA). Treated plants were transferred to a cylindrical plastic cage (30 cm in height and 15 cm in diameter) with a screen lid on top and all cages were kept under controlled conditions (27 ± 0.5°C and 14:10 h of light–dark regime) during the entire experimental period. Three independent cages were used for each strain. Negative control groups, sprayed with water plus surfactant, were included. Mortality was assessed after 1 week, and dead insects found in each cage were collected and placed in wet chambers for an additional 5 days at 26 ± 0.5°C to confirm the infection based on fungal outgrowth.

Next, the differential susceptibility of adults and nymphs of *B. pulchellus* to infection by the strains CG1105 of *B. bassiana* and IBCB425 of *M. anisopliae* was also evaluated under laboratory conditions. Cages containing *B. humidicola* plants were infested with

25 adults or fifth-instar nymphs from the colony as previously described. Plants with the insects were sprayed with a 3 mL conidial suspension (4×10^7 viable conidia mL^{-1}) of both strains, using the airbrush described above. Five independent cages were used for each of the strains and a negative control group of another five cages was sprayed with water plus surfactant. Adults and nymphs mortalities were assessed after a 10-day period of incubation. The bioassay was repeated on a different date with a different insect generation, and all the conditions and procedures followed those already described.

Identification of defensive compounds produced by *Blissus pulchellus*

To obtain the defensive compounds, groups of 20 live males, females or fifth-instar nymphs of *B. pulchellus* were collected, with eight replicates for each gender and six replicates for nymphs. The insects were immersed in 2 mL of *n*-hexane (95%) for 5 min to extract the compounds. After the extraction, each solution was filtered through a Pasteur pipette plugged with glass wool to remove any solid material or insect remnants. The resulting extracts were then concentrated using a gentle stream of nitrogen to achieve a final volume of approximately 500 µL and stored at −20°C until analysis.

The body extracts were analysed by gas chromatography (Agilent 7890A) using a 30 m × 0.25 mm ID and 0.25 µm film thickness column (DB-5MS, J&W Scientific, Folsom, CA, USA). The oven temperature was maintained at 40°C for 2 min and programmed at 15°C min^{-1} to 250°C and held for 10 min. The carrier gas was helium. The column effluent was analysed with a flame ionization detector (FID) at 270°C. One microliter of each sample was injected using splitless mode. Data were collected with ChemStation (Agilent, CA, USA). Selected volatile samples were analysed using a gas chromatograph (Agilent 7890A) coupled with an Agilent 5975-MSD mass spectrometer equipped with a quadrupole analyser, DB-5MS column (30 m × 0.25 mm ID and 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) and splitless injector with helium as the carrier gas. Ionization was by electron impact (70 eV and source temperature at 230°C). The oven temperature was maintained at 40°C for 2 min and programmed at 15°C min^{-1} to 250°C and held for 10 min. Data were collected and analysed with GC–MS ChemStation 2.1 software (Agilent, CA, USA). The compounds were identified by comparing the GC retention times and mass spectra fragmentation patterns with those of chemical standards. The compounds were quantified using calibration curves prepared with synthetic solutions of standards hexanal (97%), (*E*)-2-hexenal (98%), (*E*)-2-hexenyl acetate (98%), (*E*)-2-octenal (95%), tridecane (99%), linalool (0.0001 to 0.01 $mg.mL^{-1}$–97%), all purchased from Sigma Aldrich and (*E*)-2-octenyl acetate purchased from BedoukianBio. The compounds (2*E*,4*E*)- (2,4) hexadienyl acetate and 4-oxo-(*E*)-2-hexenal were quantified using the (*E*)-2-hexenyl acetate and (*E*)-2-hexenal curves, respectively.

Effect of defensive compounds produced by adults of *Blissus pulchellus* on conidia survival

The detrimental effect of defensive compounds produced by *B. pulchellus* on conidia germination was assessed by their exposure to the extracted compounds and by their direct contact with insect integument. In the first case, crude extracts were obtained by washing 20, 40, 60, 80 or 100 living adults in 1000 µL of *n*-hexane in glass vials for 20 min. A droplet of 20 µL of each extract was placed on the surface of PDA medium, and after complete hexane evaporation, 10 µL of a conidia suspension of *M. anisopliae* (IBCB425) or *B. bassiana* (CG1105) was inoculated onto the extract layer formed on the medium surface. Pure *n*-hexane was used as a negative control. The plates were sealed and incubated for 18 h at 25 ± 0.5°C. Germination was assessed by direct microscopic observation at 400× magnification (Nikon Eclipse Ci, Nikon Corporation, Tokyo, Japan), with germinated conidia identified as those with germ tubes longer than the width of an ungerminated conidium. The bioassay was repeated four times with independent groups of insects.

In the second experiment, groups of 20 living adults were immersed in conidia suspensions (2×10^8 viable conidia mL^{-1}) of *M. anisopliae* or *B. bassiana* for 15 s. In this case, the strains IBCB425 and CG1105 and four other strains (CG1419 and CG257 of *M. anisopliae* and CG425 and CG1420 of *B. bassiana*, see Supplementary Table S1) were used. Insects were then kept alive in petri dishes for 4 h at 25 ± 0.5°C and RH >90%. After that, conidia were retrieved from the insect surface by washing the whole group of adults into 200 µL of distilled water plus surfactant (Tween 80%–0.1% v/v) for 1 min in a vortex. A droplet of 20 µL of these suspensions was placed on PDA medium (plus streptomycin 0.5 g L^{-1}) surface and plates were incubated for 18 h. Suspensions of conidia in water without insects were used as a negative control. All the conditions and procedures for conidia germination assessment followed those already described. The bioassay was repeated three times with independent groups of insects.

For a better understanding of the insect defence mechanisms against the fungal infection, the germination of *M. anisopliae* conidia (IBCB425) on the insect forewings was evaluated under a light microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, Japan). Insects were cooled down at 4°C for 15 min to facilitate insect manipulation and wing removal. First, forewings from untreated adults were detached and kept separately in petri dishes for 6 h for natural volatilization of the defensive compounds present on insect wings. After this period, 1 µL of the conidia suspension was applied on the detached wings set on a glass slide, and the droplet was allowed to completely evaporate. Detached wings were then incubated at 25 ± 0.5°C and RH > 90% for an additional 24 h before observation under the microscope. Next, forewings of adults previously immersed in a conidia suspension and kept alive in petri dishes for 24 h were also detached at the base and placed directly onto a glass microscope slide for evaluation under the microscope. In both cases, the presence of geminated or non-germinated conidia was registered.

Comparative effect of defensive compounds produced by *Blissus pulchellus* on formulated and non-formulated conidia

Groups of 20 living adults or fifth-instar nymphs were immersed in a conidial suspension of *M. anisopliae* (IBCB425 2×10^8 viable conidia mL^{-1}) for 15 s and then kept in Petri dishes for 4 h at 25 ± 0.5°C and RH > 90%. An additional group of adults was immersed in an oil-in-water emulsion containing conidia originating from the mixture of an oil-based formulation in distilled water (1:20). The formulation was previously prepared by adding dry conidia (0.2 g) from culture plates in a mixture (9.8 g) of soybean oil (95%) and Tween 80 (5%). After that, conidia adhered to the insect surface were retrieved as previously described. A droplet of 20 μL of these suspensions was placed on the surface of PDA medium (plus streptomycin 0.5 g L^{-1}), and plates were incubated for 18 h. Suspensions of conidia in water and in oil-in-water emulsion without insects were used as negative controls. All the conditions and procedures for conidia germination assessment followed those already described. The bioassay was repeated six times with independent groups of insects.

Statistical analysis

Analyses of all the experiments were performed using R Statistical Software (R Development Core Team, 2022). The number of adults killed by the strains in the screening bioassay and the number of germinated conidia in the in vitro experiments were fitted to a generalized linear model (GLM) with binomial distribution (logit-link function). Model selection was performed to choose the best model to fit proportional data using the 'hnp' package, considering overdispersion (Moral et al., 2017). The selected models underwent analysis of variance (LRT-test). Multiple pairwise comparisons between treatments were performed with estimated marginal means at $p < 0.05$ ('emmeans' package).

To compare the quantities of defensive compounds extracted from the bodies of males, females and nymphs, the data were analysed using analysis of variance (ANOVA) followed by Tukey's post hoc test or using a *t* test ($\alpha = 0.05$). The statistical analyses were conducted using Paleontological Statistics Software (PAST, version 4.17). To determine whether the composition of defensive compound blends is specific to females, males or nymphs, principal components analysis (PCA) was applied to the multivariate data. The PCA was conducted using a correlation matrix to compare the defensive profiles among females, males and nymphs. The analysis was performed using PAST, version 4.17.

RESULTS

Susceptibility of *Blissus pulchellus* to different *Metarhizium* and *Beauveria* strains

No sign of infection by fungi was detected from the field-collected population kept in lab during the quarantine period. Differences in the total number of dead adults were detected among the fungal strains in the screening bioassay ($\chi^2 = 37.2$; df = 10; $p \leq 0.001$), but mortality rates did not surpass 45% seven days after exposure to conidia (Figure 1). The percentage of insects showing mycosis was lower than 8.5% of all treated adults. Adults and fifth-instar nymphs were equally resistant to infection by both *M. anisopliae* and *B. bassiana*. The difference in mortality was observed only between adults treated with *B. bassiana* and untreated nymphs ($\chi^2 = 25.9$; df = 5; $p = 0.023$). The average mortalities of adults and nymphs 10 days after exposure to fungal conidia were 22.0% and 19.8% for *M. anisopliae* and 26.3% and 16.9% for *B. bassiana*, respectively (Figure 2).

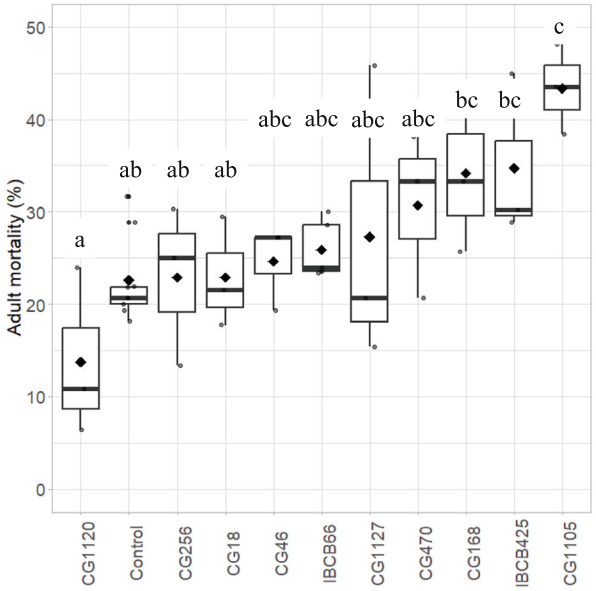

FIGURE 1 Mortality (%) of *Blissus pulchellus* adults exposed to conidia of different fungal strains at a concentration of 4×10^7 viable conidia mL^{-1} on *Brachiaria humidicola* plants after seven days of incubation at 27 ± 0.5°C and RH >85%. Each boxplot displays the mean value represented by a black diamond. Different letters on boxes indicate statistically differences in insect mortality based on pairwise comparisons (LRT-test, $p < 0.05$).

Identification of defensive compounds produced by *Blissus pulchellus*

The chemical analysis of body extracts from males and females showed minor qualitative differences in the composition of the defensive compounds, but significant quantitative differences were observed (Supplementary Figure S1). The mean total amount of defensive compounds extracted from males, females and nymphs differed significantly ($F = 4.364$, df = 2, $p = 0.028$) (Supplementary Figure S2).

Extracts from females contained significantly higher levels of defensive compounds compared to males ($p = 0.024$), but there were no significant differences between females and nymphs ($p = 0.716$), nor between males and nymphs ($p = 0.169$).

When the individual compounds were evaluated, (*E*)-2-hexenal was quantified in higher quantities in female extracts, while (*E*)-2-hexenyl acetate was more abundant in male extracts (Table 1, Figure 3). In contrast, nymphs and adults showed both qualitative and quantitative differences in their defensive compound blends. Nymph extracts presented higher amounts of hexanal and 4-oxo-(*E*)-2-hexenal; these compounds were also present in the adults' blends (Supplementary Table S2 and Figure S1). In the adult extracts, we identified compounds that were not detected in nymphs, such as (*E*)-2-hexenal, (*E*)-2-hexenyl acetate and (*E*)-2-octenyl acetate (Table 1). The PCA grouped males and females separately from nymphs and further distinguished females from males (Figure 3). The higher quantity of (*E*)-2-hexenal (C2) was primarily responsible for the separation between males and females, while nymphs were grouped due to their higher levels of tridecane (C11), 4-oxo-(*E*)-2-hexenal (C3) and hexanal (C1) (Figure 3).

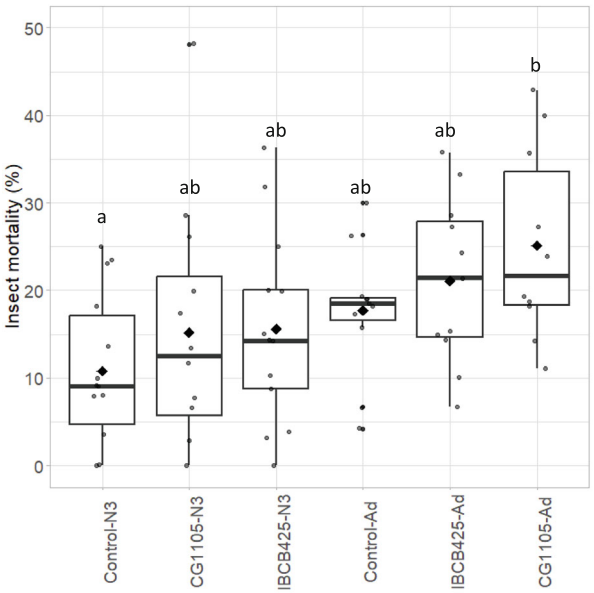

FIGURE 2 Mortality (%) of *Blissus pulchellus* adults (Ad) and fifth-instar nymphs (N3) exposed to conidia of *Beauveria bassiana* (CG1105) and *M. anisopliae* (IBCB425) at a concentration of 4×10^7 viable conidia mL^{-1} on *Brachiaria humidicola* plants after ten days of incubation at 27 ± 0.5°C and RH > 85%. Each boxplot displays the mean value represented by a black diamond. Different letters on boxes indicate statistically differences in insect mortality based on pairwise comparisons (LRT-test, $p < 0.05$).

Effect of defensive compounds produced by *Blissus pulchellus* on conidia survival

The hexanic solution containing the defensive compounds extracted from living adults and placed on PDA medium negatively affected conidia germination of both fungal strains compared to the medium without any substance ($\chi^2 = 366.8$; df = 11, 36; $p \leq 0.001$) but this effect depended on the number of insects washed. Conidia germination for both tested strains did not differ from the negative control (C-Tw) when the hexanic extract, containing the defensive compounds, obtained from 20 insects was applied to the medium (Figure 4a).

The inhibitory effect was detected when the number of adults washed in the organic solvent increased, resulting in 15.8% and 34.5% reduction in conidia germination for *M. anisopliae* and *B. bassiana* at 100 insects, respectively. The fungus *M. anisopliae* was less susceptible to the extracted compounds than *B. bassiana* (Figure 4a). The inhibitory effect was much stronger when conidia of both *M. anisopliae* and *B. bassiana* strains remained attached to the insect body for 4 h ($\chi^2 = 12{,}517$; df = 11; $p \leq 0.001$) and germination rates were lower than 20% for the different fungal strains (Figure 4b).

Defensive compounds from adults and nymphs were fungitoxic ($\chi^2 = 7514.2$; df = 4; $p \leq 0.001$). Compounds produced by nymphs

TABLE 1 Quantity of defensive compounds, in ng/bug/24 h (mean ± standard error) identified in the body extracts of adults, males and females, and 5th instar nymphs of *Blissus pulchellus*.

Compounds	RI	Females	Males	5th nymphs
Hexanal	805	717.206 ± 45.950b	628.631 ± 51.075b	6527.954 ± 699.028a
(*E*)-2-hexenal	862	16004.725 ± 836.831a	10102.025 ± 727.450b	–
4-oxo-(*E*)-2-hexenal*	968	547.228 ± 200.870b	1012.926 ± 109.191b	7609.701 ± 1077.817a
(2*E*,4*E*)-2,4-hexadienyl acetate*	1007	16.454 ± 5.151b	61.785 ± 12.684a	78.357 ± 28.024a
(*E*)-2-hexenyl acetate	1014	143.154 ± 34.718b	795.858 ± 93.414a	–
(*E*)-2-octenal	1062	599.547 ± 259.616a	315.550 ± 28.263a	84.246 ± 17.472a
Linalool	1102	99.804 ± 2.581a	101.782 ± 2.669a	105.299 ± 8.722a
Dodecane	1200	214.699 ± 11.421a	172.921 ± 14.053a	189.353 ± 36.350a
(*E*)-2-octenyl acetate	1212	126.423 ± 3.766a	123.017 ± 5.059a	–
Tridecene*	1292	Traces	Traces	Traces
Tridecane	1300	9160.551 ± 426.646ab	7457.676 ± 560.046a	11018.454 ± 1750.746b

Note: RI = retention index calculated using a DB-5 MS column.
*Tentatively identified.

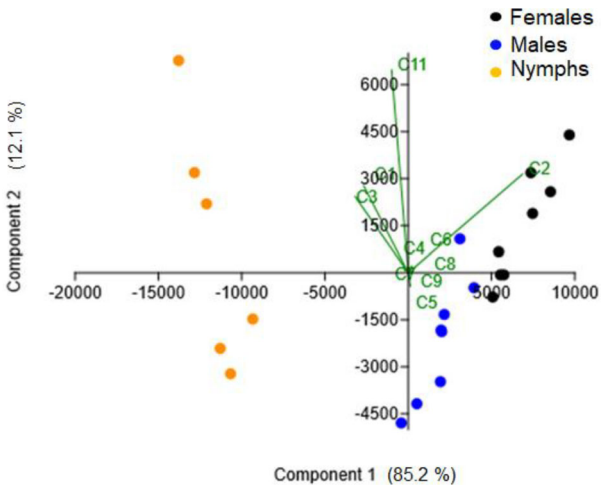

FIGURE 3 Principal component analysis (PCA) bi-plot derived from volatile organic compounds (VOCs) extracted from males, females and nymphs of *Blissus pulchellus*. Plot shows the scores of the samples from different gender and nymphs along the first and second PC and the contribution of each VOC to the first two PCs. C1—hexanal, C2—(*E*)-2-hexenal, C3—4-oxo-(*E*)-2-hexenal, C4—(2*E*,4*E*)-2,4-hexadienyl acetate, C5—(*E*)-2-hexenyl acetate, C6—(*E*)-2-octenal, C7—linalool, C8—dodecane, C9—(*E*)-2-octenyl acetate and C11—tridecane.

(Ny-Tw) were more effective in killing the fungal cells than those produced by adults, causing reductions of 93.2% and 82.0% in conidia germination, respectively (Figure 5). Formulated conidia (OD) retrieved from treated adults were affected by the defensive compounds to the same extent as unformulated conidia. The light microscopy observation of the wings detached from adults immersed in the fungal suspension showed a strong inhibitory effect, with conidia germination being nearly zero (Figure 6a,b). Differently, conidia germination on wings detached from the insect body 6 h before fungal treatment showed full conidia germination (Figure 6c,d).

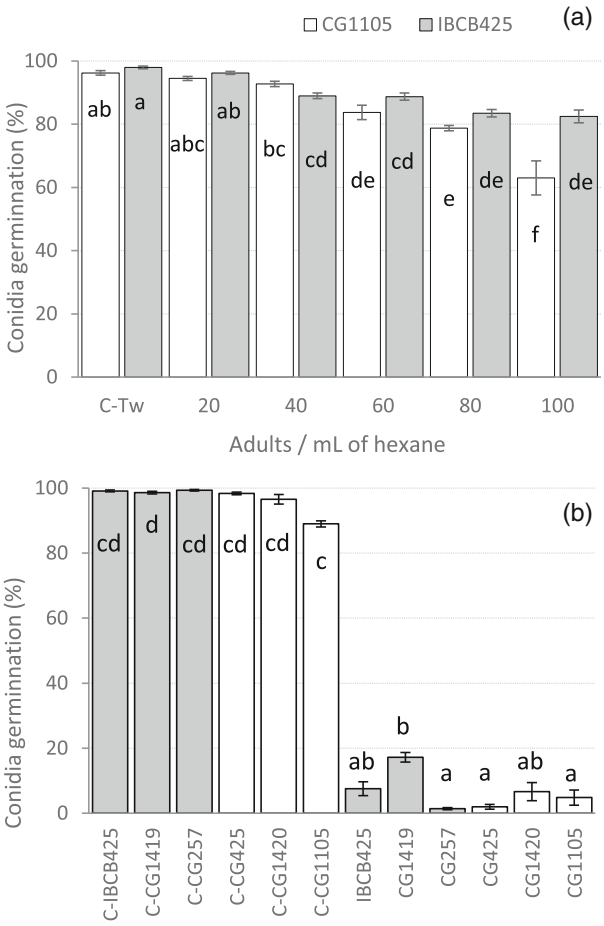

FIGURE 4 Germination (%) of conidia on PDA culture medium after 18 h of incubation at 25°C. (a) *Beauveria bassiana* CG1105 and *Metarhizium anisopliae* IBCB425 conidia exposed to different adult extracts of *Blissus pulchellus* in *n*-hexane. (b) *M. anisopliae* (grey bars) and *B. bassiana* (white bars) conidia from different strains washed from adults 4 h after inoculation with water suspension. C—control treatment (conidia not exposed to insect integument). Different letters on bars indicate statistically differences in germination based on pairwise comparisons (LRT-test, $p < 0.05$).

DISCUSSION

Defensive compounds produced by certain hemipterans are known to act against natural enemies, primarily by directly deterring predation (Moraes et al., 2008). For example, ants have been shown to have little effect in controlling populations of *Blissus*, probably due to chemical defences produced by chinch bug adults (Cherry, 2001). The presence of these allelochemicals on the insect integument plays a significant role in protecting against microbial infections. Indeed, the antifungal effects of glandular secretions from other hemipterans have been documented in previous studies (Borges et al., 1993; Lopes et al., 2015; Silva et al., 2015; Timonin, 1961; Ulrich et al., 2015). In the present study, we analysed the composition of the defensive blends produced by adults and nymphs of *B. pulchellus* and for the first time described their role in protecting the insect against infection by entomopathogenic fungi.

The primary compounds produced by *B. pulchellus* adults are also found in other hemipterans, such as the stink bugs *Nezara viridula*, *Euschistus heros*, *Oebalus poecilus* and *Diceraeus melacanthus* and the chinch bug *M. diplopterus* (Moraes et al., 2008; Okosun, 2012; Pareja et al., 2007). These hemipterans produce defensive compounds such as linear hydrocarbons (e.g., tridecane, dodecane and undecane), (*E*)-2-alkenes (e.g., (*E*)-2-hexenal, (*E*)-2-octenal and (*E*)-2-decenal) and their acetates. Although these compounds are shared among stink and chinch bugs, studies have shown that the defensive blends are species-specific (Pareja et al., 2007).

Our findings suggest that, beyond the total quantity of compounds, the presence of specific compounds at higher concentrations is more effective against fungal infections. Although nymph and male extracts contained similar total levels of defensive compounds, nymph extracts exhibited a significantly greater inhibitory effect compared to those from females and males. This suggests that aldehydes produced in significantly higher quantities by nymphs, such as hexanal and 4-oxo-(*E*)-2-hexenal, may be responsible for inhibiting fungal growth.

In contrast, acetates, which are produced exclusively by males and females, likely do not contribute to antifungal activity. Further studies should investigate the role of these specific aldehydes, as well as other aldehydes present in the body extracts.

The strong inhibitory effect observed in the germination tests after the conidia exposure to defensive compounds present on insect integument was confirmed by the low mortality rates of adults and nymphs observed in the bioassays. Our results indicate that the close and continuous contact between the fungus and the antimicrobial substances on the integument of living individuals effectively protects the chinch bug from infection. Body extracts in *n*-hexane collected from adults showed less inhibitory effect on conidia, since the defensive compounds were probably present in lower quantities on the medium after *n*-hexane evaporation. In fact, the conidia-inhibiting substances degrade or volatilize rapidly, as full germination was observed on wings that had been previously detached from adults. These observations corroborate those made by Boyle and Cutler (2012), when extract collected from dead chinch bugs did not inhibit spore germination. The oil-based formulation of *M. anisopliae* did not increase insect mortality when compared to unformulated conidia. Although most conidia remained encapsulated into oil droplets in the oil-in-water emulsion, this oily barrier did not effectively protect the cells from the defensive compounds present on insect integument. The aldehydes described in our study present miscibility in the oil phase within the emulsion and the oily coating seems to be not effective in protecting the conidia against these bioactive compounds.

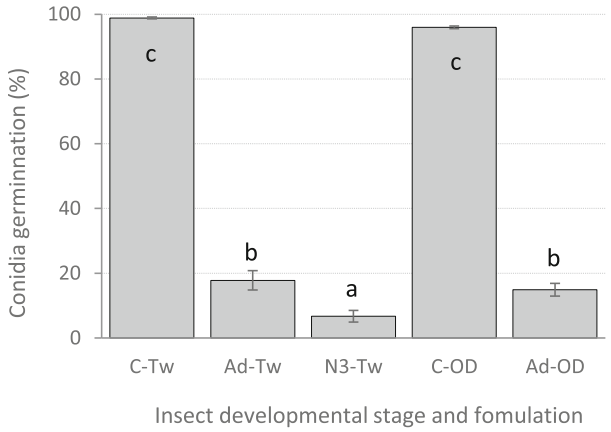

FIGURE 5 Germination (%) on PDA culture medium of *Metarhizium anisopliae* IBCB425 conidia washed from adults (Ad) or fifth-instar nymphs (Ny) of *Blissus pulchellus* 4 h after inoculation with water suspension (Tw) or oil-in-water emulsion (OD). C—control treatment (conidia not exposed to insect integument). Different letters on bars indicate statistically differences in germination based on pairwise comparisons (LRT-test, $p < 0.05$).

Nymphs were as resistant to infection by *M. anisopliae* and *B. bassiana* as the adults. The low susceptibility of adults and nymphs of *B. pulchellus* to infection by different *M. anisopliae* and *B. bassiana* strains was previously reported by Samuels and Coracini (2004). Although a strain of *B. bassiana* (ARSEF792) originally obtained from naturally infected *B. leucopterus* was able to kill around 78% of the adults in that study, mortality ranged from only 19% to 47% for nine other strains, even at a concentration of conidia more than 10 times higher than the one used in our bioassays. Other studies report high

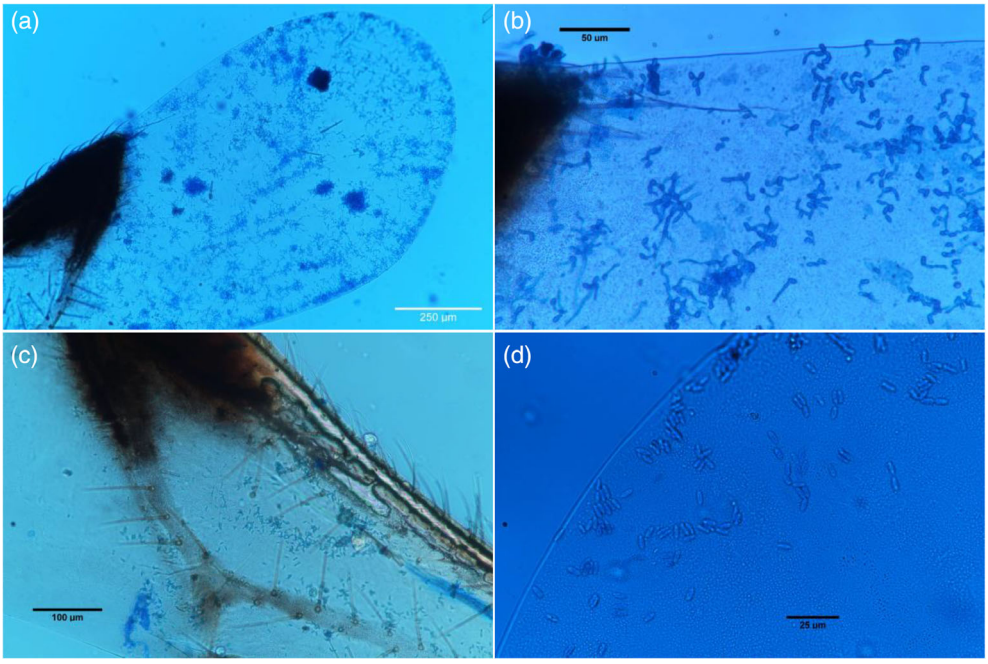

FIGURE 6 Forewings from *Blissus pulchellus* treated with *Metarhizium anisopliae* IBCB425 conidia. A, B—wings detached from adults 6 h before being treated with a conidia suspension showing full germination after 24 h-period in a moistened chamber (magnification of 100 × and 400 ×). C, D—wings detached from adults immersed in a conidia suspension and kept for a 24 h-period in petri dishes showing ungerminated conidia (magnifications of 200 × and 600 ×).

mortality levels of chinch bug adults and nymphs in the laboratory, but only when insects were exposed continuously to high concentrations of conidia (Boyle & Cutler, 2012; Krueger et al., 1991; Ramoska, 1984). The need for high doses of fungal propagules to produce satisfactory levels of infection may be a bottleneck for a wider adoption of mycopesticides against chinch bugs in the field. This may explain why the strategy adopted in the United States was interrupted during the late nineteenth century. The strategy aimed to initiate epizootics by inoculating an endemic 'white fungus' (*B. bassiana*) in areas heavily infested by *Blissus* (Billings & Glenn, 1911).

Recent outbreaks of chinch bug populations in northern Brazil (Fidelis et al., 2021) and areas of pasture in Mato Grosso and Rondônia states are likely to become more frequent with rising temperatures and drought, impacting forage production in those regions. Strategies for biologically controlling chinch bugs in pastures must account for the insect population dynamics across different pasture varieties and growing conditions throughout the year. While high concentrations of conidia may be necessary to effectively reduce insect infestation, large-scale applications tend to be economically prohibitive. The multigenerational aggregation behaviour of chinch bugs (Addesso et al., 2012) leads to the formation of insect clusters and grass patches showing visible symptoms of attack (Baxendale et al., 1999; Lima et al., 2021; Reinert et al., 2011). Therefore, mycopesticide applications can be targeted to the patches and their marginal areas, allowing the use of higher and more effective doses in specific spots where the population is concentrated. Identifying fungal species or strains that are naturally resistant to insect defensive compounds, along with developing formulations that protect conidia, will be crucial for optimizing future mycopesticide applications.

AUTHOR CONTRIBUTIONS

Isis Carolina Souto Oliveira: Investigation; conceptualization; writing – review and editing. **Maria Carolina Blassioli-Moraes:** Investigation; conceptualization; writing – original draft; funding acquisition; formal analysis; writing – review and editing. **Caio Augusto Rosado Torres:** Conceptualization; investigation; writing – review and editing. **Giancarlo Catafesta:** Conceptualization; investigation; writing – review and editing. **Elisangela Gomes Fidelis:** Project administration; funding acquisition; writing – review and editing. **Raul Alberto Laumann:** Writing – review and editing. **Miguel Borges:** Investigation; writing – review and editing. **Rogerio Biaggioni Lopes:** Writing – original draft; funding acquisition; investigation; conceptualization; writing – review and editing; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

Data used in this manuscript are available from the Zenodo Digital Repository. https://doi.org/10.5281/zenodo.15576262.

ETHICS STATEMENT

This study does not contain any studies with human participants or vertebrate animals, and no ethical approval is required.

INFORMED CONSENT

All authors reviewed and approved the final version of this manuscript and consented for its publication.

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REFERENCES

Addesso, K.M., Mcauslane, H.J. & Cherry, A.N. (2012) Aggregation behavior of the southern chinch bug (Hemiptera: Blissidae). *Environmental Entomology*, 41(4), 887–895. Available from: https://doi.org/10.1603/EN11145

Baxendale, F.P., Heng-Moss, T.M. & Riordan, T.P. (1999) *Blissus occiduus* (Hemiptera: Lygaeidae): a chinch bug pest new to buffalograss turf. *Journal of Economic Entomology*, 92(5), 1172–1176. Available from: https://doi.org/10.1093/jee/92.5.1172

Billings, R.H. & Glenn, P.A. (1911) Results of the artificial use of the white fungus disease in Kansas. USDA Bureau of Entomology, Bulletin No 107, 58pp.

Borges, M., Leal, S.C.M., Tigano-Milani, M.S. & Valadares, M.C.C. (1993) Efeito do feromônio de alarme do percevejo verde, *Nezara viridula* (L.) (Hemiptera: Pentatomidae), sobre o fungo entomopatogênico *Metarhizium anisopliae* (Metsch.) Sorok. *Anais da Sociedade Entomologica do Brasil*, 22, 505–512. Available from: https://doi.org/10.37486/0301-8059.v22i3.883

Boyle, D. & Cutler, G.C. (2012) Effects of insect activity, soil, and cuticular factors on virulence of *Beauveria bassiana* toward *Blissus leucopterus hirtus*. *Journal of Pest Science*, 85, 505–512. Available from: https://doi.org/10.1007/s10340-012-0453-y

Cherry, R. (2001) Interrelationship of ants (Hymenoptera: Formicidae) and southern chinch bugs (Hemiptera: Lygaeidae) in Florida lawns. *Journal of Entomological Science*, 36(4), 411–415. Available from: https://doi.org/10.18474/0749-8004-36.4.411

Costa, R.G. (1945) Uma praga das gramíneas. *Boletim Agronômico*, 9, 39–40.

Fidelis, E.G., Oliveira, R., Bendahan, A.B., Carvalho, J.S., Valério, J.R. & Henry, T.J. (2021) First occurrence and population dynamics of *Blissus pulchellus* (Hemiptera: Blissidae) in Brazil: a new pest of pastures

in Roraima. *Revista Brasileira de Entomologia*, 65(2), 1–6. Available from: https://doi.org/10.1590/1806-9665-RBENT-2020-0096

Krueger, S.R., Nechols, J.R. & Ramoska, W.A. (1991) Infection of chinch bug, *Blissus leucopterus leucopterus* (Hemiptera: Lygaeidae), adults from *Beauveria bassiana* (Deuteromycotina: hyphomycetes) conidia in soil under controlled temperature and moisture conditions. *Journal of Invertebrate Pathology*, 58(1), 19–26. Available from: https://doi.org/10.1016/0022-2011(91)90157-L

Lima, A., Valada, T., Caetano, M.F., Franco, J.C. & Ramos, A.P. (2021) First record of the lawn chinch bug *Blissus insularis* barber (Hemiptera: Blissidae) in Europe. *Phytoparasitica*, 49, 539–545. Available from: https://doi.org/10.1007/s12600-021-00903-1

Lopes, R.B., Laumann, R.A., Blassioli-Moraes, M.C., Borges, M. & Faria, M. (2015) The fungistatic and fungicidal effects of volatiles from metathoracic glands of soybean-attacking stink bugs (Heteroptera: Pentatomidae) on the entomopathogen Beauveria bassiana. *Journal of Invertebrate Pathology*, 132, 77–85. Available from: https://doi.org/10.1016/j.jip.2015.08.011

Lord, J.C. (2005) From Metchnikoff to Monsanto and beyond: the path of microbial control. *Journal of Invertebrate Pathology*, 89(1), 19–29. Available from: https://doi.org/10.1016/j.jip.2005.04.006

Moraes, M.C.B., Pareja, M., Laumann, R.A. & Borges, M. (2008) The chemical volatiles (semiochemicals) produced by Neotropical stink bugs (Hemiptera: Pentatomidae). *Neotropical Entomology*, 37, 489–505. Available from: https://doi.org/10.1590/S1519-566X2008000500001

Moral, R.A., Hinde, J. & Demétrio, C.G.B. (2017) Half-normal plots and overdispersed models in R: the hnp package. *Journal of Statistical Software*, 81(10), 1–23. Available from: https://doi.org/10.18637/jss.v081.i10

Okosun, O.O. (2012) Chemical ecology and eco-physiology of the grain chinch bug, Macchiademus diplopterus (Distant) (Hemiptera: Lygaeidae: Blissidae), a phytosanitary pest of South African export fruit. Thesis Uni. Stellenbosch, 1–108.

Pareja, M., Borges, M., Laumann, R.A. & Blassioli-Moraes, M.C. (2007) Inter- and intraspecific variation in defensive compounds produced by five neotropical stink bug species (Hemiptera: Pentatomidae). *Journal of Insect Physiology*, 53(7), 639–648. Available from: https://doi.org/10.1016/j.jinsphys.2007.04.004

R Core Team. (2022) *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Ramoska, W.A. (1984) The influence of relative humidity on *Beauveria bassiana* infectivity and replication in the chinch bug, *Blissus leucopterus*. *Journal of Invertebrate Pathology*, 43, 389–394. Available from: https://doi.org/10.1016/0022-2011(84)90085-5

Reinert, J.A., Chandra, A. & Engelke, M. (2011) Susceptibility of genera and cultivars of turfgrass to southern chinch bug *Blissus insularis* (Hemiptera: Blissidae). *Florida Entomologist*, 94, 158–163. Available from: https://doi.org/10.1653/024.094.0206

Reis, P.R.A., Costa, J. & Lobato, L.C. (1976) *Blissus leucopterus* (Say) (Hemiptera: Lygaeidae), nova praga das gramíneas introduzida no Estado de Minas Gerais. *Anais da Sociedade Entomologica do Brasil*, 5(2), 241–242. Available from: https://doi.org/10.37486/0301-8059.v5i2.83

Samuels, R.I. & Coracini, D.L.A. (2004) Selection of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for the control of *Blissus antillus* (Hemiptera: Lygaeidae). *Scientia Agricola*, 61(3), 271–275. Available from: https://doi.org/10.1590/S0103-90162004000300005

Samuels, R.I., Coracini, D.L.A., Santos, C.A.M. & Gava, C.A.T. (2002) Infection of *Blissus antillus* (Hemiptera: Lygaeidae) eggs by the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Biological Control*, 23(3), 269–273. Available from: https://doi.org/10.1006/bcon.2001.1009

Shimer, H. (1867) Notes on *Micropus* (*Lygarus*) *leucopterus* say, ("The chinch bug") with an account of the great epidemic disease of 1865 among insects. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 19, 75–80.

Silva, R.A., Quintela, E.D., Mascarin, G.M., Pedrini, N., Lião, L.M. & Ferri, P.H. (2015) Unveiling chemical defense in the rice stalk stink bug against the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, 127, 93–100. Available from: https://doi.org/10.1016/j.jip.2015.03.009

Snow, F.H. (1891) Chinch bugs: experiments in 1890 for their destruction in the field by the artificial introduction of contagious diseases. *Biannual Report Kansas State Board of Agriculture*, 12, 184–188.

Sosa-Gómez, D.R., Boucias, D.G. & Nation, J.L. (1997) Attachment of *Metarhizium anisopliae* to the southern green stink bug *Nezara viridula* cuticle and fungistatic effect of cuticular lipids and aldehydes. *Journal of Invertebrate Pathology*, 69, 31–39. Available from: https://doi.org/10.1006/jipa.1996.4619

Spike, B.P., Wilde, G.E., Mize, T.W., Wright, R.J. & Danielson, S.D. (1994) Bibliography of the chinch bug, *Blissus leucopterus leucopterus* (Say) (Hemiptera: Lygaeidae) since 1888. *Journal of the Kansas Entomological Society*, 67, 116–125.

Thomas, C. (1879) *The chinch-bug, its history, characters, and habits, and the means of destroying it or counteracting its injuries*. Washington: United States Entomological Commission, Bulletin N° 5, p. 44.

Thompson, J.G. (1909) The rise and decline of the wheat growing industry in Wisconsin. Bulletin of the University of Wisconsin N° 292. *Economic and Political Science Series*, 3, 295–544. Available from: https://doi.org/10.1086/251680

Timonin, M.I. (1961) Effect of volatile constituents of *Scaptocoris talpa* champ. On the growth of soil fungi. *Plant and Soil*, 14, 323–334. Available from: https://doi.org/10.1007/BF01666291

Ulrich, K.R., Feldlaufer, M.F., Kramer, M. & St. Leger, R.J. (2015) Inhibition of the entomopathogenic fungus *Metarhizium anisopliae* sensu lato in vitro by the bed bug defensive secretions (E)-2-hexenal and (E)-2-octenal. *BioControl*, 60, 517–526. Available from: https://doi.org/10.1007/s10526-015-9667-2

Valério, J.R., Reis, P.R. & Lima, O.G. (2015) Percevejo-das-gramíneas, *Blissus leucopterus*? In: Vilela, E.F. & Zucchi, R.A. (Eds.) *Pragas Introduzidas no Brasil: insetos e ácaros*. Piracicaba: FEALQ, pp. 708–719.

Valério, J.R., Vieira, J.M. & Valle, L.C.S. (1999) The occurrence of *Blissus antillus* Leonard (Hemipera: Lygaeidae: Blissinae) in Tangola pasture in Mato Grosso do Sul state, Brazil. *Anais da Sociedade Entomologica do Brasil*, 28(3), 527–529. Available from: https://doi.org/10.1590/S0301-80591999000300020

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1. Supporting Information.

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