A Test of Immune Priming in the Kissing Bug *Rhodnius pallescens* (Hemiptera: Reduviidae) against the Entomopathogenic Fungus *Beauveria bassiana* (Hypocreales: Cordycipitaceae) in Panama[1]

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Abstract The assumption that the invertebrate immune system lacks memory and specificity has changed over time: many studies now indicate that a primary exposure of the host to a pathogen increases its resistance to a subsequent lethal challenge, a phenomenon known as immune priming. One group of insects in which immune priming has been little investigated is the hematophagous triatomine bugs. Herein, we tested the capability of the kissing bug *Rhodnius pallescens* Barber (Hemiptera: Reduviidae; hereafter kissing bugs), the vector of Chagas disease, to resist entomopathogenic fungi. Laboratory kissing bugs free of *Wolbachia* and *Trypanosoma* spp. as well as kissing bugs collected from the wild were used for tests with the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae). Against laboratory kissing bugs, the fungus remained virulent for 94 d, indicating long-term viability. Kissing bugs collected from the wild that were exposed to a nonlethal dose of the fungus did not show increased survival against a lethal dose compared with controls inoculated with the lethal dose. However, kissing bugs inoculated with a nonlethal dose had higher levels of total phenoloxidase than control kissing bugs. Although the fungus activates the immune system of the kissing bugs, other variables may influence survival in the face of infection. Moreover, the lethality of the same strain was lower against wild kissing bugs, suggesting that the presence of symbionts or parasites influence the fungus–triatome (host) interaction. This work is one of the few studies that have investigated the fungus–host interaction in terms of immune priming in a hematophagous insect of public health importance. Implications are discussed.

Key Words *Beauveria*, Chagas disease, entomopathogenic fungus, immune priming, triatomine

It is now well accepted that immunological memory is widely distributed in invertebrates (Lanz-Mendoza and Contreras-Garduño 2022). The basic concept is that primary exposure of the host to a nonlethal dose of the pathogen increases its resistance to a subsequent lethal challenge, a phenomenon known as immune

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priming (Cooper and Eleftherianos 2017). The benefits of the immune priming have been reported in the context of within and across developmental stages and at the transgenerational level (Contreras-Garduño 2016, Khan et al. 2016, Sheehan et al. 2020, Tetreau et al. 2019).

Despite the popularity of immune priming in recent years, experimental data on insect immune priming come from relatively few species (Contreras-Garduño 2016, Sułek et al. 2021), making it difficult to assess whether the occurrence is universal or restricted to specific combinations of hosts and pathogens or experimental conditions and also making it difficult to understand the mechanistic basis of the phenomenon (Rowley and Powell 2007, Sułek et al. 2021).

Therefore, there is a need to study immune priming in other insects to better understand the ecological and evolutionary basis across species with different immune strategies (Prakash and Khan 2022). Immune priming has been documented in several insect species exposed to bacteria or bacterial molecules (Burciaga et al. 2023, Rosengaus et al. 1999, Roth et al. 2009, Sadd and Schmid-Hempel 2006), protozoa (Rodrigues et al. 2010), and viruses (Tidbury et al. 2010). As concerns experimentation with entomopathogenic fungi, the number of studies and evidence are limited, with cases in termites (Rosengaus et al. 1999), *Drosophila melanogaster* Meigen (Pham et al. 2007), *Lasius niger* Ruzsky ant queens (Gálvez and Chapuisat 2014), and *Galleria mellonella* (L.) larvae (Sheehan et al. 2021). However, lack of evidence for immune priming against fungi is also reported in *G. mellonella* (Vertyporokh and Wojda 2020) and in workers and queens of *Formica selysi* Seifert (Gálvez and Chapuisat 2014, Reber and Chapuisat 2012). In some cases, the evidence is conflictive, such as in queens of *Crematogaster scutellaris* (Olivier) exposed to the fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin that can elicit an increased resistance in the offspring but without developing an increased immune response themselves (Bordoni et al. 2019). The study of immune priming against fungi in nonmodel insects may provide a deeper understanding of the phenomenon. A group of insects for which little research has been done on immune priming is the hematophagous kissing bugs (Reduvidae: Triatominae).

Immunological studies and immune priming tests conducted with hematophagous arthropods are from studies on arachnids, such as ticks and mites, due to their medical importance, with evidence of upregulation (Matsuo et al. 2004, Nakajima et al. 2001) and increased survival controlled by molecular pathways that are apparently unique to ticks (Shaw et al. 2017). Work evaluating immune priming in hematophagous insects has been focused on mosquitoes, responding to virus (Blagrove and Barribeau 2021, Vargas et al. 2020), bacteria (Kulkarni et al. 2021), and protozoa (Ramirez et al. 2015).

Triatomines provide a novel assemblage of hematophagous species for the study of immune responses in insects. Carmona-Peña et al. (2021) highlighted the need to study whether immune memory occurs in triatomines, particularly against different strains and species of *Trypanosoma* (Carmona-Peña et al. 2022). Another crucial element would be to investigate whether the triatomines show some kind of memory against pathogens such as entomopathogenic fungi. Given the medical importance of triatomines as vectors of Chagas disease (Stevens et al. 2011), this work offers a line of research that could provide valuable information to develop tools for the control of their populations. Understanding the entomopathogenic fungi–triatomine relationship and its interaction with influential

factors might provide insight into their population control by the development of new strains, blocking the transmission of disease-causing agents, and prevent the emergence of populations resistant to parasites, pathogens, and pesticides (Ortiz-Urquiza et al. 2015). Herein, we tested the immune priming response in *Rhodnius pallescens* Barber (Hemiptera: Reduviidae; hereafter kissing bugs) against the entomopathogenic fungus *B. bassiana* by measuring survival and total phenoloxidase in the hemolymph of kissing bugs that were exposed to a low dose of the fungus and later exposed to a lethal dose, compared with the control kissing bugs.

Materials and Methods

Experiment 1: laboratory kissing bugs. *Rhodnius pallescens* is one of the main vectors of *Trypanosoma cruzi* (Chagas) in Panama and the only triatomine transmitter of *Trypanosoma rangeli* (Tejera) in the country (Rodriguez and Loaiza 2017). This experiment was intended to be a test of immune priming, but logistical difficulties caused one of the treatments to be missed (control-*Beauveria*, see Experiment 2). However, we considered that the data obtained from this experiment are valuable and worth presenting.

We used kissing bugs that were free of infection by *T. cruzi* and the bacteria *Wolbachia*, a line that has been established at the Centro de Investigaciones Parasitarias (CIDEP) of the University of Panama. Kissing bugs were fed every 21 d with blood from domestic chickens.

Beauveria bassiana was cultured on potato dextrose agar and incubated at room temperature (25–27°C). The conidial solution was prepared by scraping the culture surface and placing it in 2 ml of sterile Tween 80 (0.01%) aqueous solution. The concentration of the fungus was determined by counting conidia with a Neubauer chamber. For the inoculations, almost half of the kissing bugs were inoculated on the thorax with 2 μl of a nonlethal dose of the fungal solution (1×10^2 conidia/ml) and the other half of the kissing bugs were treated with 2 μl of the control solution. Seven days later, almost half of the kissing bugs inoculated with the fungus were inoculated with a lethal concentration of the fungal solution (1×10^4 conidia/ml) and the control kissing bugs were inoculated again with the control solution. All kissing bugs were placed individually in cylindric plastic containers (460 ml) with a piece of damp cotton inside the container, and the container was kept in the dark in an environmental chamber. Survival of the kissing bugs was monitored daily for 94 d.

Experiment 2: wild kissing bugs. Because of an insufficient number of laboratory kissing bugs, we decided to conduct the immune priming test with wild kissing bugs. We collected kissing bugs in Trinidad de las Minas (08°47.02924′, −080°00.00644′) in July and August 2019 by using live-baited adhesive traps following Noireau et al. (2002), but we used T-shaped polyvinyl chloride (PVC) connectors instead of straight shapes. We used the same strain of the fungus as used in Experiment 1. Half of the kissing bugs were inoculated with 2 μl of a nonlethal dose of the fungal solution (9×10^2 conidia/ml) on the thorax, and the other half was inoculated with 2 μl of the control solution. Seven days later, half of each group was inoculated with a high dose (9×10^7 conidia/ml; *Beauveria*-*Beauveria*, control-*Beauveria*) and the other half was inoculated with the control solution (control-control, *Beauveria*-control). Each kissing bug was placed individually in a

plastic container as described in Experiment 1. The survival of the kissing bugs was monitored daily for 40 d.

Experiment 3: total phenoloxidase. We used wild kissing bugs to estimate the levels of total phenoloxidase (phenoloxidase plus prophenoloxidase). We took kissing bugs from the "priming" and control groups for the measurements, 3 d after the inoculation. The head and the last three segments of the abdomen were removed. The thorax and the remainder of the abdomen were macerated in 60 μl of 0.2 M sodium cacodylate. The sample was vortexed for 1 min and centrifuged for 10 min at 4°C at 4,000 rpm. Next, 15 μl of the supernatant was frozen at −20°C until later use. Subsequently, 4 μl of the supernatant was used, mixed with 10 μl of phosphate-buffered saline, and 50 μl of trypsin was added to this mixture in microplate wells; the wells were left to rest for 5 min at room temperature. Next, 10 μl of L-DOPA was added and the absorbance at 492 nm was measured every 10 s for 50 min at 30°C (Castella et al. 2009).

Statistical analysis. Survival data from Experiments 1 and 2 were analyzed with a survival analysis from the survival package (survreg function, R Core Team 2023), specifying treatment and sex as factors, with full interaction. The model was simplified in terms of Akaike's Information Criterion. We used the pairwise_survdiff function for pairwise comparisons, with a Bonferroni–Holm correction.

To determine the levels of total phenoloxidase, we obtained the slope of the reaction curve during the linear phase of the reaction (maximum velocity). The results were analyzed using a logarithmic transformation due to the lack of normality in the data, and a linear regression was used that included as factors the treatment (*Beauveria* versus control), sex, and interaction of these variables.

Results

Experiment 1: laboratory kissing bugs. Kissing bugs exposed twice to the control solution survived the entire 94-d experiment (Fig. 1, control-control), whereas kissing bugs that were subjected to a nonlethal dose during the first exposure and subsequently to the control solution showed a lower survival (Fig. 1, *Beauveria*-control versus control-control; log-rank test, $P < 0.0001$). By contrast, individuals that were exposed twice to the fungus also showed a lower survival than control kissing bugs (Fig. 1, *Beauveria-Beauveria* versus control-control; log-rank test, $P < 0.0001$). Double exposure to the fungus induced a higher mortality than a single inoculation (Fig. 1, *Beauveria-Beauveria* versus *Beauveria*-control, $P = 0.003$). Sex of the kissing bug had no effect on survival ($\chi^2 = 0.47$, $P = 0.50$), and there was no interaction between sex and treatment ($\chi^2 = 0.60$, $P = 0.75$), indicating that both sexes responded similarly to all treatments.

Experiment 2: wild kissing bugs. Inoculation with the lethal dose of the fungus did not reduce the survival of kissing bugs initially inoculated with the control (Fig. 2, control-control versus control-*Beauveria*, log-rank test, $P = 0.4$) or kissing bugs initially inoculated with a nonlethal dose of the fungus (Fig. 2, *Beauveria*-control versus *Beauveria-Beauveria*, $P = 0.5$). Control kissing bugs tended to show higher survival than kissing bugs exposed to the nonlethal dose against the lethal dose, but this difference was no longer significant after the correction (Fig. 2,

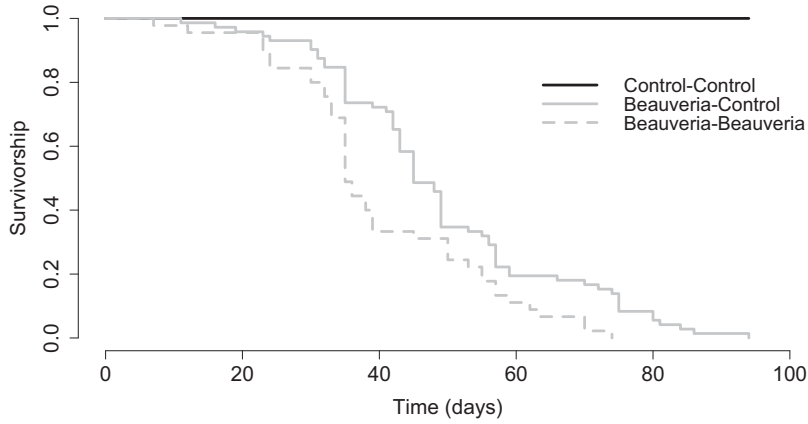

Fig. 1. Survival curves of laboratory kissing bugs (*Rhodnius pallescens*) after two inoculations with the control solution (control-control, $n = 95$), a first inoculation with the fungus and then the control (*Beauveria*-control, $n = 72$), or double inoculation with the fungus (*Beauveria*-*Beauveria*, $n = 45$).

control-*Beauveria* versus *Beauveria*-*Beauveria*; log-rank test, $P = 0.06$). Males tended to die faster than females; however, this difference was not statistically significant ($\chi^2 = 3.6$, $P = 0.06$).

Experiment 3: total phenoloxidase. Kissing bugs inoculated with a nonlethal dose of the fungus had significantly ($t = 2.1$, $P = 0.04$) higher levels of total phenoloxidase than the control kissing bugs (Fig. 3). There was no effect of sex ($t = 0.8$, $P = 0.41$) and no interaction between sex and treatment ($t = -1.3$, $P = 0.19$).

Discussion

We found no evidence that *R. pallescens* obtains a benefit in terms of survival because of an immune priming response after exposure to a nonlethal dose of the pathogenic fungus *B. bassiana*. In fact, the two experiments that we conducted strongly indicate that an initial inoculation with a nonlethal dose is deleterious for the kissing bugs when facing the fungus a second time. The lower mortality of wild *R. pallescens* than the laboratory-reared *R. pallescens* (free of *Wolbachia* and *Trypanozoma*) suggests that symbionts or other environmental factors influence their resistance to the fungus. However, infection by *T. cruzi* did not influence the rate of infection success by *B. bassiana* for a Colombian population of *R. pallescens* (Agudelo and Moreno 1997). Moreover, the effect of sex on the survival of the kissing bugs remains unclear because there was a trend of higher resistance for female wild kissing bugs, which would follow Bateman's principle of immunity (Rolff 2002). Yet, in some insects, females are more susceptible to *B. bassiana* than males, for example, *D. melanogaster* (Shahrestani et al. 2018). Moreover, diet can influence this sexual dimorphic response (McKean and Nunney 2005). However, there was no effect of sex for laboratory kissing bugs free of *Wolbachia*

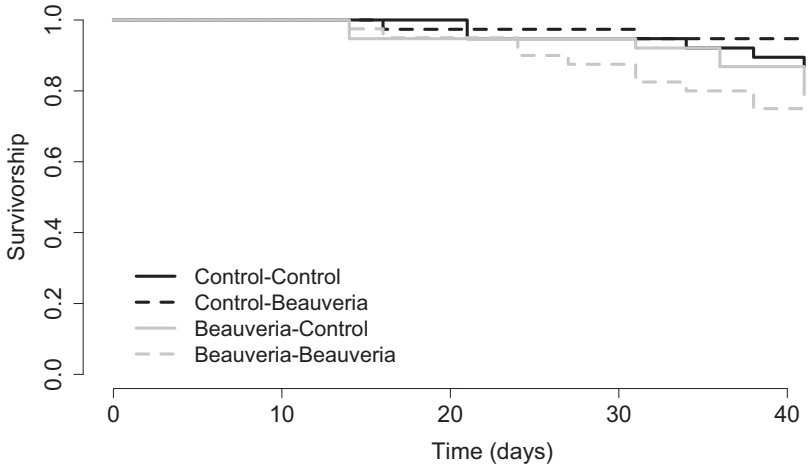

Fig. 2. Immune priming test with wild kissing bugs (*Rhodnius pallescens*). Survival curves after two inoculations with the control solution (control-control, $n = 38$), a first inoculation with the control and then a lethal dose of the fungus (control-*Beauveria*, $n = 38$), a first inoculation with a nonlethal dose of the fungus and then the control (*Beauveria*-control, $n = 38$), and double inoculation with the fungus: first nonlethal and then lethal (*Beauveria-Beauveria*, $n = 40$).

and *Trypanozoma*, again suggesting that symbionts may influence a potential sexual dimorphism in resistance to the fungus. Symbiont-driven sexual dimorphism in the immune response occurs in *Drosophila*, and *Wolbachia* mediates a stronger protection in males against *Pseudomonas aeruginosa* (Gupta et al. 2017). Further experiments might elucidate the effect of sex on the immune response of triatomines and how sex interacts with other variables (e.g., *Wolbachia*, *Trypanosoma*, diet, age).

Similar to our results, high mortality of laboratory-reared Colombian *R. pallescens* against *B. bassiana* has been reported by Pineda Gutierrez et al. (2003) and Saldarriaga et al. (2005); however, these two studies used higher doses (10^5–10^8 conidia/ml) than our dose (10^4), highlighting the potential large geographic variation in virulence of the fungus. A striking result was that the fungus remained virulent until 94 d under the laboratory conditions; however, further studies in the field are needed because some strains of *B. bassiana* remain virulent for a few weeks after application in the field (Daud et al. 2019). Moreover, it is likely that variation in susceptibility to the fungus (Wang et al. 2020) occurs across Panamanian populations of *R. pallescens*, perhaps in part explaining the variation between laboratory and wild kissing bugs in our study.

Despite that the survival experiment does not provide evidence of immune priming, the increased level of total phenoloxidase suggests that some form of immunization occurs. In *G. mellonella* larvae, nonlethal dose injections of *Candida albicans* (C.-P. Robin) Berkhout induced phenoloxidase activity, but injection of

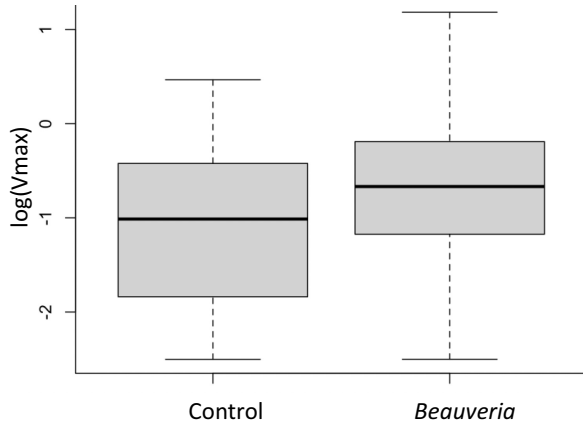

Fig. 3. Total phenoloxidase levels (measured as maximum speed [Vmax]) in samples of kissing bugs (*Rhodnius pallescens*) inoculated with a nonlethal dose of the entomopathogenic fungus *Beauveria* ($n = 22$) and with the control solution ($n = 22$).

the lethal dose resulted in strong inhibition of this enzyme after 24 h (Vertyporokh and Wojda 2020). Still, levels of phenoloxidase do not necessarily correlate with survival against a pathogen (Adamo 2004, Mucklow et al. 2004, González-Santoyo and Córdoba-Aguilar 2012, Kasianov et al. 2017), highlighting that disease resistance and immunity assays may not always correlate, depending on the host–pathogen combination (for review, see Adamo 2004). Multiple immunity assays may be required to improve our understanding. Moreover, production of phenoloxidase for triatomines may be more complex if its production can be influenced by the species of *Trypanosoma* infecting the host (Mello et al. 1995), a variable that remained uncontrolled for our wild kissing bugs that can be infected by two *Trypanosoma* species (Sousa 2002).

Another potential explanation may involve the period of immunization (7 d) being too extended; thus, the phenoloxidase levels had already decreased and provided no significant defense against the infection. Alternatively, some antimicrobial peptides (AMPs) that contribute to defense against fungal infection may not have yet been produced at sufficient levels at the time of the lethal challenge (day 7). For example, in the kissing bug *Triatoma infestans* Klug, expression of a gene (TiPPO) regulating production of prophenoloxidase (the precursor of phenoloxosidase) peaked at day 9 after inoculation with *B. bassiana* and two other genes of the humoral immune system peaked at 6–12 d (Lobo et al. 2015). This delayed response may be an adaptation to the slower invasion and replication processes of pathogenic fungi (Salcedo-Porras and Lowerberger 2021); therefore, we may have missed the time of maximal AMP levels. Further work should evaluate different times for measuring immune components and application of the lethal challenge to investigate this possibility.

Overall, given its virulence, apparent long viability, and apparent lack of a immune priming response from the host, *B. bassiana* seems a good candidate for

biological control of *R. pallescens*, as with applications used against other triatomines (Carmona-Peña et al. 2021, Garcia 2013, Pedrini et al. 2009). In addition, the sublethal dose of the fungus caused slow, steady death rates and it would be interesting to investigate potential effects on the kissing bugs, such as reducing feeding rates and fecundity (e.g., Blanford et al. 2012, Ondiaka et al. 2015, Scholte et al. 2011) or preventing the transmission of *Trypanosoma* parasites across kissing bugs, similar to that found for other systems (Blanford et al. 2005). More work is needed to understand how multiple pathogenic bacteria, fungi, and trypanosomes drive the use of multiple AMPs in triatomines while not affecting their essential microbial symbionts (Salcedo-Porras and Lowerberger 2021). Our work strongly points at the need to consider multiple factors while studying immune priming in triatomines, such as the presence of the *Trypanosoma* parasite, the symbiont *Wolbachia*, and the potential effect of sex, among other factors.

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