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Major Article

Behavioral fever response in *Rhodnius prolixus* (Reduviidae: Triatominae) to intracoelomic inoculation of *Trypanosoma cruzi*

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Abstract

Introduction: Behavioral fever is a response to infections with microorganisms observed in some poikilothermic animals. *Rhodnius prolixus* is involved in the transmission of two parasites: *Trypanosoma cruzi* (pathogenic for humans and transmitted in feces) and *Trypanosoma rangeli* (non-pathogenic for humans, pathogenic for *Rhodnius* and transmitted by the bite of an infected individual). Only *T. rangeli* is found in the hemolymph of *Rhodnius* as it travels to the salivary glands. **Methods:** To study vector-parasite interactions, we evaluated possible behavioral fever responses of *R. prolixus* to intracoelomic inoculation with *T. cruzi* or *T. rangeli*. Temperature preferences of fifth-instar nymphs of *R. prolixus* were evaluated after inoculation with *T. rangeli* KP1(+), KP1(-), *T. cruzi* I, or the Trypanosome culture medium. Four different fixed temperatures (25, 30, 35, and 40°C) in two simultaneous experiments (enclosed and free-moving insects) were evaluated. Free-moving insects were marked daily according to their temperature preferences on each of the 15 days after inoculation. Numbers of insects in each temperature shelter and daily mortality were compared with those enclosed shelters of different temperatures. **Results:** *Rhodnius prolixus* inoculated with both strains of *T. rangeli* and with the trypanosome culture medium showed preferences for the lowest temperatures (25°C). However, *R. prolixus* inoculated with *T. cruzi* I showed significant preferences for temperatures around 35°C. **Conclusions:** This is the first known investigation to demonstrate a behavioral fever response in *R. prolixus* injected intracoelomically with *T. cruzi* I.

Keywords: American trypanosomiasis. *Rhodnius prolixus*. *Trypanosoma rangeli*. *Trypanosoma cruzi*. Behavioral fever.

INTRODUCTION

In endothermic animals, fever is a common internal response to overcome microorganism infections[1]. However, ectothermic animals can only raise their internal body temperature to reduce microorganism infections by moving to warmer areas in a classic behavioral response called *behavioral fever*[1] [2] [3]. Evidence that insects can become feverish was first observed in the cockroach *Gromphadorhina portentosa* injected with an *Escherichia coli* suspension[4]. Studies over the past 25 years[5] [6] [7] [8] [9] have shown that a febrile response is not only possible, but widespread among ectotherms[10]. However, to our knowledge, there is only one previous study testing behavioral fever in blood-sucking insects involved in parasite transmissions[11].

Triatominae (Hemiptera: Reduviidae) are blood-sucking insects that are important vectors in the transmission of *Trypanosoma cruzi*, the causative agent of American trypanosomiasis or Chagas disease[12]. Chagas disease is a very complex anthropozoonosis that is widely distributed in 21 Latin-American countries, where it affects approximately 7.7 million people, with approximately 12,500 deaths and approximately 41,200 new cases each year[13]. In addition, *Trypanosoma rangeli* (apathogenic to vertebrate hosts) is also frequently found and transmitted by triatomines in Latin-America[14]. Interestingly, while *T. cruzi* is apathogenic for triatomines, *T. rangeli* is considered pathogenic to these insects[15].

In triatomines, *T. cruzi* never leaves the digestive tract system and is transmitted through contaminated feces[16]. Conversely, *T. rangeli* always leaves the digestive tract to travel to the salivary glands and is transmitted through the bite of an infected individual[14] [17]. According to Zingales et al.[18], six groups of *T. cruzi* are recognized (*T. cruzi* I to VI), while for *T. rangeli* only two groups have been defined [*T. rangeli* KP1(-) and *T. rangeli* KP1(+)][19].

In Colombia, the main vector of Chagas disease is *Rhodnius prolixus* and *T. cruzi* I and *T. rangeli* KP1(+) and KP1(-) are widely distributed[19] [20]. A close association of *R. prolixus* with *T. rangeli* KP1(+) has been observed, as it travels to the salivary glands through the hemolymph. In contrast, *T. rangeli* KP1(-) has been observed to be more closely associated with

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Rhodnius belonging to the pallescens group[17] [21]. Recently, a trypanolytic factor acting against *T. rangeli* KP1(-) was found in the hemolymph of *R. prolixus*[17] [22].

Thermopreferences in triatomines, such as *Rhodnius*, have been studied in several species, and are a dynamic process influenced by daily rhythms and starvation levels[23] [24] [25] [26] [27]. In *Triatoma infestans* and *T. brasiliensis*, daily temperature preferences vary across a broad range[23] [25], while in *Panstrongylus megistus* and *R. prolixus*, the range of daily temperature preferences varies only slightly[24] [27]. Lower temperature preferences in triatomines during the photophase and with increasing starvation would avoid unnecessary waste of water and nutrients[24].

The thermopreferences of Triatomines and the interaction between two *Trypanosoma* species with different developmental cycles inside *Rhodnius* represents a model to test the behavioral fever response against microorganisms present in the hemolymph of *R. prolixus*. We intracoelomically inoculated *T. cruzi* (not present in the hemolymph) and *T. rangeli* (present in the hemolymph). Our objectives were: 1) To test whether *R. prolixus* is able to adapt thermopreferences to develop a behavioral fever strategy; 2) to test whether behavioral fever depends on specific associations with microorganisms and the body parts they occupy in *R. prolixus*, and 3) to analyze the impact of environmental temperature on the survival of intracoelomically infected *R. prolixus* to evaluate the adaptive value of behavioral fever.

METHODS

Insects

Rhodnius prolixus were captured from wild populations in 1979 and have been maintained since as a laboratory colony. We used 1,800 fifth-instar nymphs nourished with live hen's blood every 15 days and reared at 27 ± 2°C, 75 ± 10% relative humidity, under a 12/12h light/dark schedule. Nymphs were separated into five treatments for the experiments.

Strains of *Trypanosoma*

Three strains of trypanosomes were evaluated in the experiment: a) *T. rangeli* KP1(+), isolated from domestic *R. prolixus* and maintained for 21 cycles in the laboratory; b) *T. rangeli* KP1(-), isolated from wild *R. prolixus* and maintained for 23 cycles in the laboratory; and c) *T. cruzi* I, isolated from wild *R. prolixus* and maintained for 20 cycles in the laboratory. All strains were maintained in Tobie culture medium[28] under the same laboratory conditions (temperature 25°C and 70% relative humidity).

Treatments

Five treatments were evaluated daily for 15 days: Control 1: Non-inoculated insects, Control 2: Insects inoculated only with Tobie culture medium, Experiment 1: *T. cruzi* I in Tobie culture medium, Experiment 2: *T. rangeli* KP1(+) in Tobie culture medium, and Experiment 3: *T. rangeli* KP1(-) in Tobie culture medium.

For each treatment including controls, 360 nymphs were evaluated. Nymphs used in control 2 and experiment 1 to 3 were held ventral side up on a flat surface and inoculated intracoelomically between the last fourth abdominal sternites with a tuberculine syringe. Each insect was inoculated with approximately 10µl of Tobie culture medium with a concentration of 1.32×10^4 trypanosomes/µl (determined with a Neubauer chamber or hemocytometer).

Experimental arena and shelters

All treatments were conducted on a square slide of superboard (1m × 1m × 2cm) surrounded by transparent acrylic walls 10cm in height and covered with vaseline to prevent the insects from climbing out. A square (1m × 1m × 3mm) of transparent acrylic covering the experimental arena was used to maintain constant temperature and relative humidity during the experiments. On the experimental arena, four metallic plates (20cm × 20cm) were placed at equal distances from the walls and from each other. Electric heaters were placed below each metallic plate and connected to power supplies through separately controlled thermostats to supply constant and independent temperature to each plate. Experimental temperatures were 25°C, 30°C, 35°C, and 40°C measured with thermometers (-10 to 80°C ± 0.1). A constant light source (Philips Genie 14W warm white) in the center of the arena was placed 9cm from the acrylic cover to provide 24h illumination to the arena (**Figure 1**). The light source induced insects to search for a shelter to avoid lights[29].

Two rectangular (10cm × 20cm × 5cm) and unconnected shelters were placed on each of the thermal plates. In total, eight shelters were provided for each treatment, four (shelters A to D in **Figure 1**) maintained 60 nymphs each at a constant temperature, while the other four (shelters E to H in **Figure 1**) were open to both lateral sides and allowed 120 free-moving insects to choose one of the four temperatures available.

The number of free-moving insects inside each shelter was counted daily at 18:00h, and individuals were marked on the legs according to their preferred temperature.

Dissections and parasitemia

Daily dissections of two insects from each of the closed shelters (A to D **Figure 1**) were conducted at 24h post-inoculation. Salivary glands of nymphs infected with *T. rangeli* KP1(+), salivary glands and hindguts (rectal ampulla) of *R. prolixus* infected with *T. rangeli* KP1(-), and hindguts (rectal ampulla) of *R. prolixus* infected with *T. cruzi* I were dissected in Phosphate Buffered Solution pH = 7.2 according to standard methodology[30]. Average numbers of parasites were determined daily with a Neubauer chamber or hemocytometer using a light microscope at 400 × magnification.

Data analysis

The effect of treatments and temperatures on mortality of insects enclosed in shelters A to D and the average number of insects in shelters E to H after 15 days post-inoculation were analyzed with two-way analysis of variance (ANOVA), to determine the effect of each variable independently, and the interaction between them. We assumed a mixed-design ANOVA model with one fixed effect factor (treatment: *T. cruzi* or *T. rangeli* inoculation) and one random effect factor (temperature).

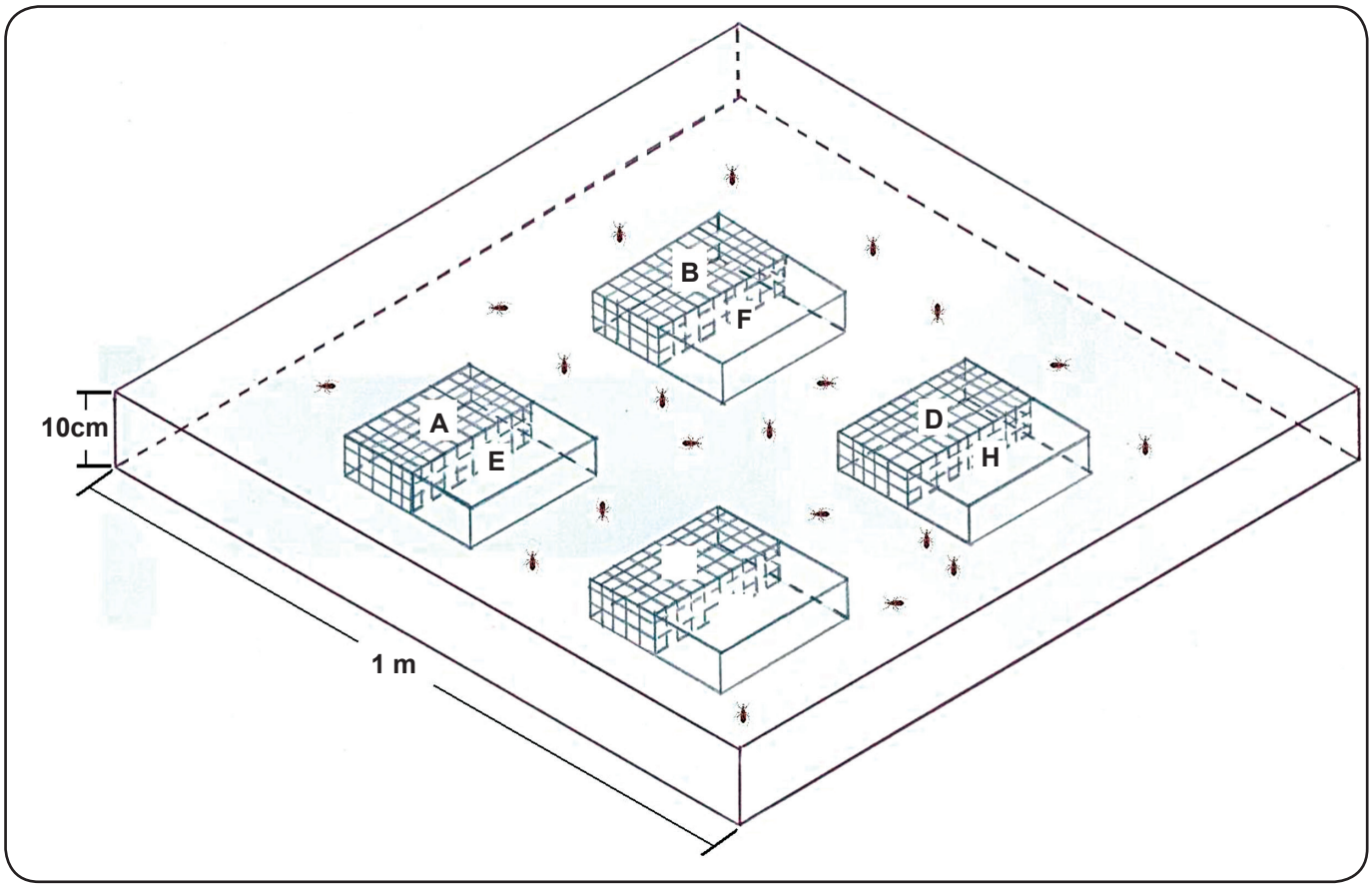

FIGURE 1. Experimental arena with shelters. **Shelters A to D:** housed insects continually enclosed at a constant temperature. **Shelters E to H:** were open and free to access by free-moving fifth-instar nymphs of *Rhodnius prolixus*

Post-hoc multiple comparisons using the Scheffé test were conducted to evaluate the effect of treatments and temperature on the survival of *R. prolixus*[31]. All statistical analyses were performed with the software Statgraphics Centurion XVI.

RESULTS

Rhodnius prolixus maintained at constant temperatures for 15 days (shelters A to D in **Figure 1**) only showed the presence of *T. rangeli* KP1(+) in salivary glands after the third day post-inoculation (**Figure 2**). The average number of parasites detected in salivary glands increased until ten days post-inoculation and was higher in insects maintained in shelters at 25°C (**Figure 2**). No trypanosomes were found in the salivary glands or rectal ampullae of insects inoculated with *T. rangeli* KP1(-) or *T. cruzi* I.

Rhodnius prolixus kept at constant temperatures for 15 days (shelters A to D in **Figure 1**) showed statistically significant differences in mortality between the five treatments tested ($p = 0.015$, F-ratio = 7.4, df = 4 two-way ANOVA), between the temperatures tested ($p = 0.028$, F-ratio = 5.2, df = 3 two-way ANOVA), and a significant interaction between treatments and temperatures ($p = 0.038$, F-ratio = 4.2, df = 1 two-way ANOVA). Post-hoc analysis showed only statistical significances in mortalities of control *R. prolixus* (non-inoculated or inoculated with Tobie medium) between the lower temperatures (25 and 30°C) and higher temperatures (35 and 40°C) ($p < 0.05$ Scheffé test). Insects inoculated with *T. rangeli* KP1(-) showed statistical differences only between 35°C and the other temperatures tested ($p < 0.03$ Scheffé test), and insects inoculated with *T. cruzi* showed statistical differences in mortality between all temperatures tested, except when comparing 30 and 40°C ($p < 0.05$ Scheffé test). No significant differences were observed between the temperatures in insects inoculated with *T. rangeli* KP1(+) ($p > 0.05$ Scheffé test). All together, these results confirm that mortalities higher than 10 insects found in both controls at 25°C and 30°C is suggesting that factors additional to hunger and inoculation procedures may have caused mortality of the triatomines (**Figure 3**).

Two interactive effects of temperature and treatment on results considering mortality are of especial interest: a reduction in mortality in *R. prolixus* inoculated with *T. cruzi* and *T. rangeli* KP1(-) at 35°C ($p < 0.05$ Scheffé test), and, an increase in mortality at 35°C in insects inoculated with *T. rangeli* KP1(+) compared to the control ($p < 0.05$ Scheffé test). This suggested that *R. prolixus* maintained at 35°C were possibly able to control a parasite infection, with the exception of those inoculated with *T. rangeli* KP1(+) (**Figure 3**). A temperature of 40°C still had some effect on parasites; however, it appears that the effect of

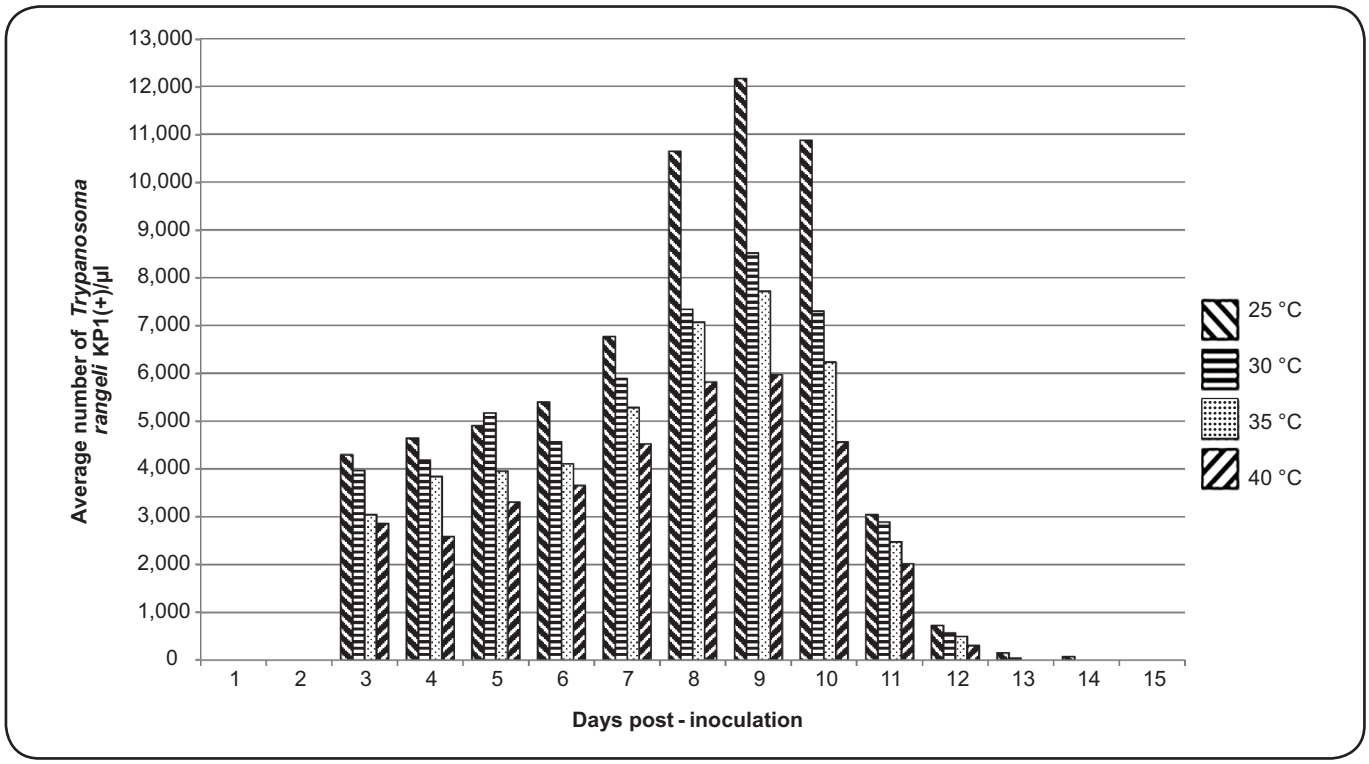

FIGURE 2. Average number of *Trypanosome rangeli* KP1(+) counted daily in salivary glands of *Rhodnius prolixus* maintained at a constant temperature for 15 days.

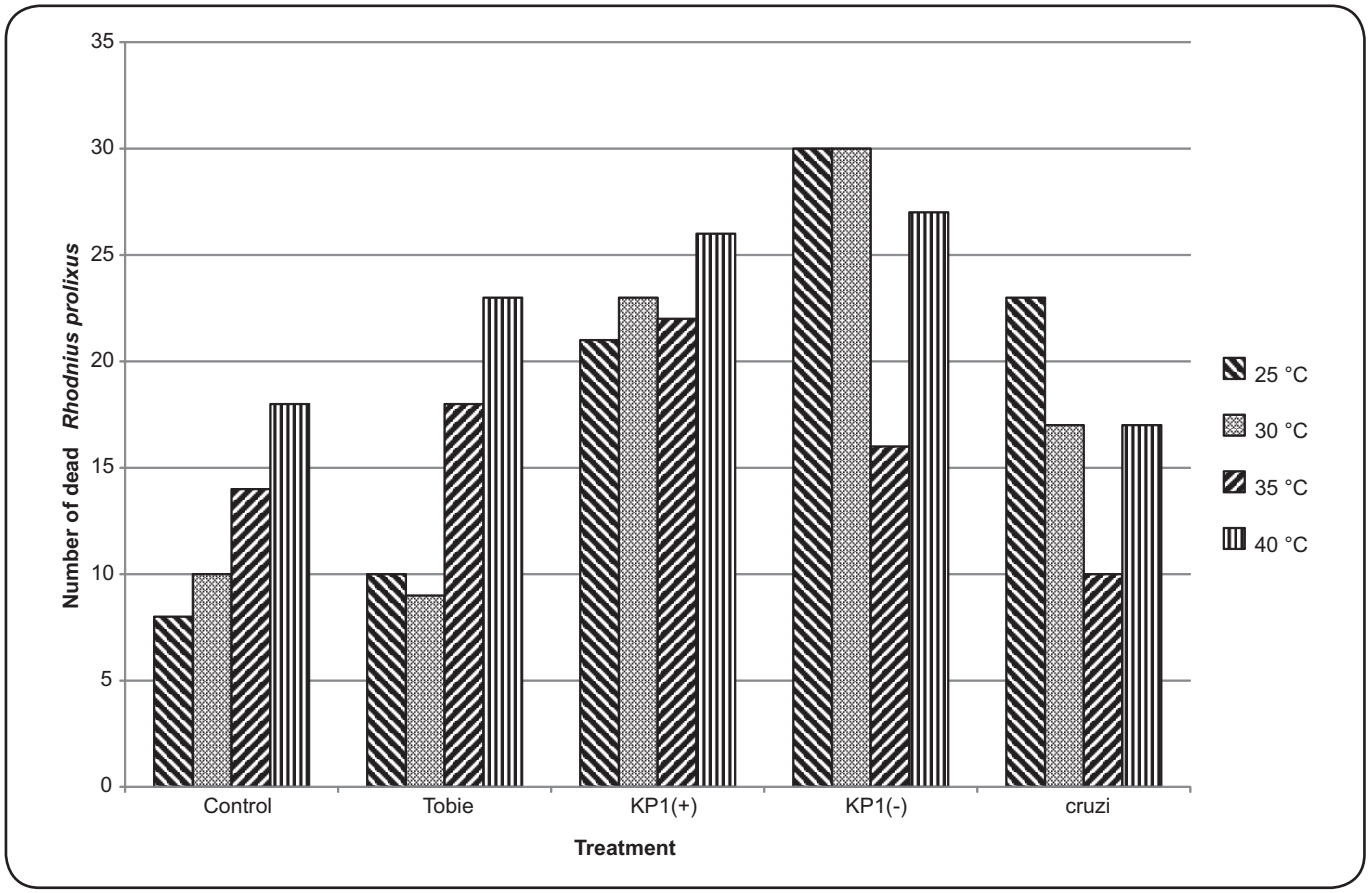

FIGURE 3. Total number of dead *Rhodnius prolixus* after 15 days maintained at a constant temperature. .

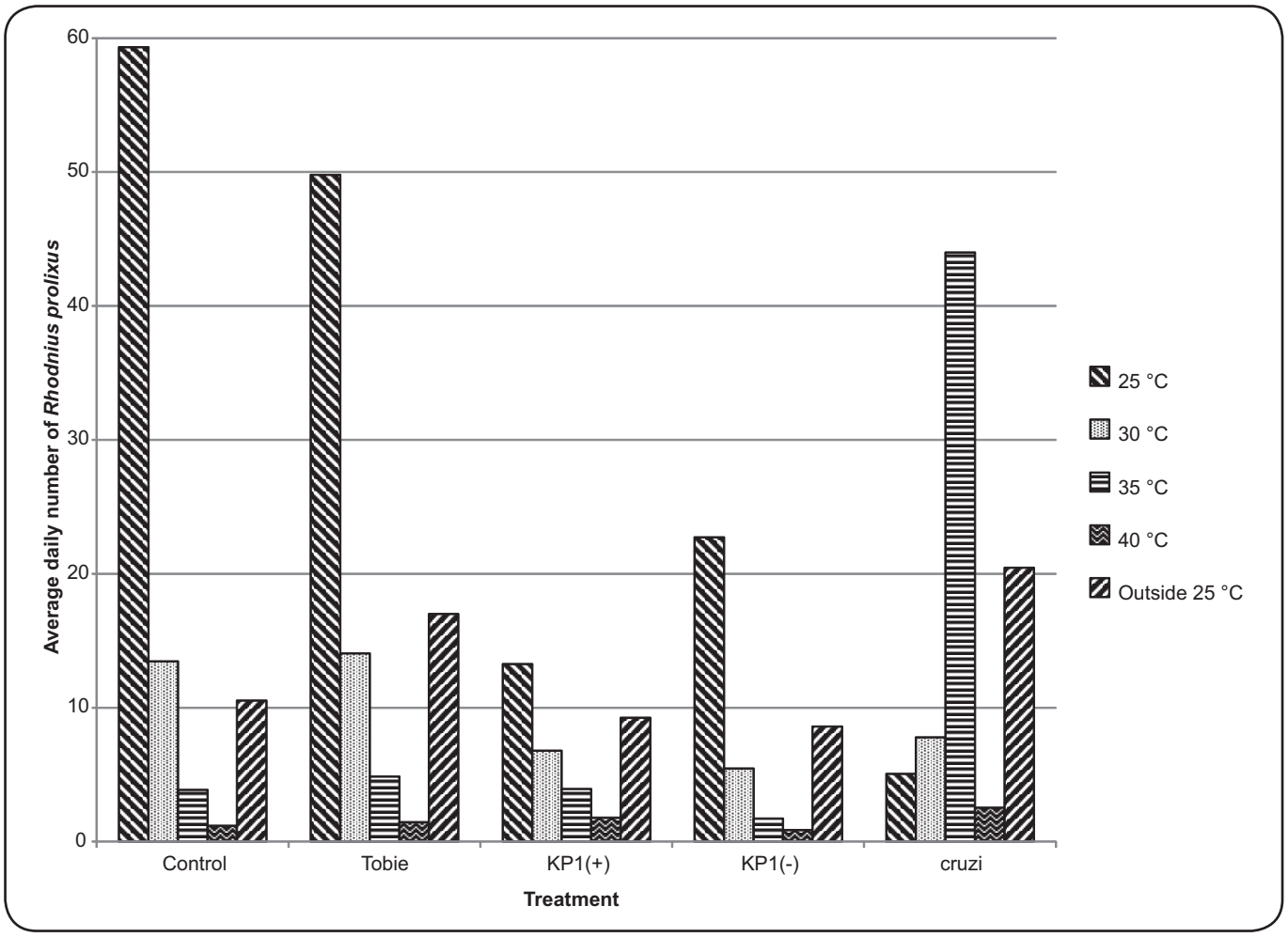

FIGURE 4. Average daily number of living *Rhodnius prolixus* observed over the 15 days in each evaluated temperature.

temperature on the insects was stronger and again, an increase in mortality was observed in all treatments (**Figure 3**).

These results indicated that *Rhodnius prolixus* inoculated only with *T. rangeli* KP1(-) or *T. cruzi* and that were free to move between shelters with different temperatures searched for shelters with temperatures around 35°C to overcome the infection.

Interestingly, insects that were free to move between shelters (E to H in **Figure 1**) showed preferences related to treatment ($p = 0.04$, F-ratio = 4.1, df = 4 two-way ANOVA), temperature ($p = 0.02$, F-ratio = 5.8, df = 3 two-way ANOVA), and a significant interaction between both factors ($p = 0.042$, F-ratio = 4.0, df = 1 two-way ANOVA) (**Figure 4**). Post-hoc analysis showed statistical significant preferences in *R. prolixus* in control experiments (non-inoculated or inoculated with Tobie medium) for shelters at 25°C ($p < 0.02$ Scheffé test), 30°C, or 25°C outside ($p < 0.05$ Scheffé test), and that insects consistently avoided shelters at 35°C or 40 °C (**Figure 4**). Insects inoculated with *T. rangeli* KP1(+) or KP1(-) also showed a preference for shelters at 25°C ($p < 0.05$ Scheffé test); however, on average more nymphs inoculated with *T. cruzi* were found in shelters at 35°C ($p < 0.03$ Scheffé test) or at 25°C outside ($p < 0.04$ Scheffé test) (**Figure 4**).

Finally, a post-hoc comparison of treatments and temperatures showed that *R. prolixus* inoculated with *T. cruzi* significantly preferred shelters at 35°C in comparison to all other treatments at the same temperature ($p < 0.05$ Scheffé test).

In summary, *R. prolixus* inoculated with *T. rangeli* [KP1(+) or KP1(-)] showed higher mortality rates and resulted in fewer live insects after 15 days on average, compared to non-inoculated insects, and insects inoculated with Tobie or with *T. cruzi* (**Figure 4**).

DISCUSSION

Insects can respond to microbial and parasite infections by altering their thermoregulatory behavior and developing behavioral fever. An increase in body temperature above normal thermal levels from moving to warmer areas has been shown to favor host survival in a number of host-pathogen interactions[4][5][6][7][8][9], including *Rhodnius prolixus-Trypanosoma* interactions (**Figure 3** and **Figure 4**).

Thomas et al.[11] demonstrated behavioral fever in *Xenopsylla cheopis* infected with *Yersinia pestis*. Results showed that fleas infected with *Yersinia* bacteria did not exhibit preferences for high temperatures.

Triatominae insects exposed to constant light sources exhibit a strong, negative, phototactic response[29]. Our experiments used this phototactic response to induce infected *Rhodnius prolixus* to move to one of the four dark shelters with different temperatures available in the experimental arena.

We hypothesized that *R. prolixus* inoculated with *T. rangeli* KP1(+) and *T. rangeli* KP1(-) should behaviorally search for shelters with higher temperatures to overcome the infection with parasites that are pathogenic for triatomines[14][15]. However, our experiments with nymphs maintained at constant temperatures showed that non-inoculated triatomines or insects inoculated with Tobie presented a higher mortality at 35°C and 40°C (**Figure 3**). Therefore, individuals must achieve a balance between temperatures high enough to reduce the infection, but not survival.

Trypanosoma cruzi and *T. rangeli* in Tobie culture medium under laboratory conditions are incubated at 25°C to 28°C[32]. Constant temperature above 30°C is lethal for trypanosomes in culture medium (unpublished data from our group), suggesting that shelters at 35°C could provide the ideal solution to reduce infection with minimum impact on survival.

The interaction between temperature and treatments presented two possible results depending on the parasite that was inoculated: a synergistic effect between temperature and parasites (in this case an increase in mortality was expected in triatomines exposed to higher temperatures compared to those exposed to lower temperatures); and, an inhibitory effect between temperature and parasites (in this case lower mortality was expected in triatomines exposed to higher temperatures than in those exposed to lower temperatures). We regarded the second possibility as the true behavioral fever response.

Our results showed both kinds of possible responses (**Figure 3**). *Rhodnius prolixus* showed an increase in mortality when *T. rangeli* KP1(+) and higher temperature were positively associated (See **Figure 3**). Conversely, a reduction in mortality of *R. prolixus* was observed when insects were inoculated with *T. rangeli* KP1(-) or *T. cruzi* exclusively at 35°C (**Figure 3**).

The differences in responses observed between *R. prolixus* inoculated with *T. rangeli* KP1(+) and KP1(-) can be partially explained by coevolutionary processes. *T. rangeli* KP1(-) is a trypanosome circulating in the Andes mountains and is mainly associated with *Rhodnius* of the pallescens group, while *T. rangeli* KP1(+) circulates to the east of the Andes in close association with *Rhodnius* of the prolixus group[21][33].

Given the results from *R. prolixus* maintained at a constant temperature, we expected that bugs free to move to a shelter would prefer 35°C, but only if they were inoculated with *T. rangeli* KP1(-) or *T. cruzi*.

Experiments with free-moving triatomines showed preferences for shelters at 25 and 30°C in both non-inoculated *R. prolixus* and those inoculated with Tobie medium (**Figure 4**). These results were unsurprising as this is the temperature range maintained in the laboratory breeding conditions and corresponds to the temperatures recommended for triatomines care and maintenance procedures[34].

However, when nymphs of *R. prolixus* were inoculated with *T. cruzi* they showed behavioral fever responses by moving to shelters at 35°C (**Figure 4**).

A possible explanation for the behavioral fever response observed in *R. prolixus* could be the route of inoculation. Intracoelomic inoculation introduces *T. cruzi* (a non-pathogenic *Trypanosoma* for triatomines) directly into the hemocoel, where *T. cruzi* is not present under natural conditions[35]. In other words, the presence of a foreign cell in the hemocoel produced a behavioral fever response to overcome the infection and reduce mortality (**Figure 3**).

Why was behavioral fever not observed in *R. prolixus* inoculated with *T. rangeli* KP1(-)? A possible explanation is a recently discovered trypanolytic protein in *R. prolixus* that can function as an additional barrier against parasites. This molecule is responsible for inhibiting the development of *T. rangeli* KP1(-), making *R. prolixus* resistant to this kind of parasite strain[17]. In addition, it has been demonstrated that behavioral fever in insects is a specific response expressed toward a small subset of pathogens[8].

From an immunological point of view, behavioral fever responses to a subset of pathogens can be expected as different survival of *T. rangeli* and *T. cruzi* in the hemolymph of *R. prolixus* has been observed after inoculation into the hemocoel[35][36]. Differences in the survival of parasites have been related to an induction of lysozymes, agglutinating, prophenoloxidase, and hemocyte activity[36][37]. For instance, the presence of *T. rangeli* in the hemocoel of *R. prolixus* activates the prophenoloxidase system, phagocytosis, hemocyte microaggregation, superoxide and nitric oxide activity, and eicosanoid biosynthesis[38].

This study of *R. prolixus* inoculated intracoelomically with Trypanosomes showed that behavioral fever should only be expected in those cases where hosts have not coevolved to make the appropriate response against parasites. As with other insects[4][5][6][7][8][9], *R. prolixus* is only able to develop behavioral fever against unknown microorganisms in the hemolymph.

Future experiments with other triatomine species, including different routes of infection, such as ingesting blood infected with trypanosomes, would confirm the effect of behavioral fever on *Trypanosoma* spp. It would also be interesting to evaluate the effect of behavioral fever on natural populations of triatomines exposed to pathogens that are potentially involved in biological control, such as fungi[9][39][40][41][42][43] and viruses[44][45].

Conflict of interest

The authors declare that there is no conflict of interest.

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