

# Temperature increase impairs recognition among nestmates in the social wasp *Polybia paulista* H. von Ihering, 1896 (Vespidae: Polistinae: Epiponini)

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**Abstract.** Cuticular hydrocarbons (CHCs) of most terrestrial arthropods primarily serve as a protective barrier against desiccation and infection. Throughout evolution, these compounds have acquired another fundamental function: the exchange of signals during interactions between nestmates. However, even though cuticular hydrocarbons perform a dual function in social insects, little is known about the effect(s) of one function on the other in social insects, and no study has evaluated this relationship in social wasps. Therefore, the present study tests the hypothesis that the level of aggressiveness presented during induced encounters between nestmates of *Polybia paulista* who were subjected to different conditions temperature is different than between nestmates who remained under the same temperature conditions. If the hypothesis is confirmed, it is likely because the cuticle of the wasps that had been exposed to temperature variation adjusted to these conditions leading them not to recognize the cuticular chemical signature of their colony. To test this hypothesis, workers were exposed to temperature variation in a BOD chamber and then subjected to encounters with workers who were maintained at a constant temperature of 24°C. We also used control groups to evaluate the effect of isolation alone among the groups. According to our results, our hypothesis was confirmed, the level of aggressiveness presented between nestmates who were exposed to temperature variation and those who remained at 24°C was significantly higher than the levels of aggressiveness presented between nestmates who remained isolated but under constant temperature during the same period, in some cases, it was similar to the aggressiveness presented in encounters between wasps from different colonies. During these encounters, wasps performed alarm behavior, bites, and stings not seen during encounters between wasps that remained under the same temperature, but in isolated groups. The lack of aggressive behavior under isolated conditions indicates that isolation had no effect on chemical recognition signature. These results suggest that temperature variation may have caused some change in the cues that allow recognition between nestmates. On the other hand, these results were not caused by isolation or stress generated by the study design and difference in the CHC profile of workers, as described in the literature, is consistent with our results.

**Keywords.** Pheromones; Cuticular hydrocarbons; Vespidae; Wasp aggression; Nestmate recognition; Chemical communication.

## INTRODUCTION

Communication among individuals of a group is essential for the formation of stable and highly organized social structures (Turillazzi, 2012). In social insects, the predominant form of com-

munication is chemical in nature (Hölldobler & Wilson, 1990; Le Conte & Hefetz, 2008). In other words, communication is based on the activity of chemical messenger molecules, or pheromones (Hölldobler & Wilson, 1990; Billen, 2006; Le Conte & Hefetz, 2008). Pheromones are substances se-

creted by an animal, released into the environment, and meant to cause a specific behavioral reaction in the individual receiving it from the same species (Karlson & Butenandt, 1959).

The chemical compounds that serve as pheromones are produced in the exocrine glands of insects and secreted in a way that can be detected by another individual to induce some specific behavioral response (Billen, 2011). Among the compounds containing such information are those composed of long carbon chains found on the surface of the insect's cuticle (Howard, 1993). These cuticular compounds are hydrocarbons (CHCs), consisting of a complex mixture of linear alkanes, branched alkanes and alkenes (Brown *et al.*, 1991; Howard & Blomquist, 2005; Hefetz, 2007), ranging from 20 to 40 carbons (Lockey, 1988).

The linear alkanes consist of saturated carbon chains and are suggested to be more related to waterproofing by the higher adhesion caused by strong van der Waals bonds, while branched alkanes and alkenes have branches and double bonds that can disrupt tight aggregation between molecules (Menzel *et al.*, 2017) and seem to be important in the communication system (Chung & Carroll, 2015). On the other hand, some compounds with intermediate melting temperatures and volatility, such as some alkenes and branched alkanes, may be involved in both waterproofing and chemical communication (Chung & Carroll, 2015).

Studies have shown that the cuticle undergoes a significant change in its compounds in response to temperature (Gibbs *et al.*, 1997; Menzel *et al.*, 2018; Michelutti *et al.*, 2018; Sprenger *et al.*, 2018; Duarte *et al.*, 2019) in order to adjust to new conditions. In *Polybia paulista* H. von Ihering, 1896, the percentage of linear alkanes increases, while the percentage of branched alkanes decreases, along an increasing temperature gradient. Tolerance to temperature seems to be mediated by the reactive change in chemical profile of the cuticle (Michelutti *et al.*, 2018).

CHCs play a primary role in nestmate recognition and ensure that the work expended by the colony members does not benefit members of other colonies (Richard & Hunt, 2013). The high specificity of CHCs allows social wasps to use these chemical cues to identify nestmates and non-nestmates (Gamboa *et al.*, 1986; Downs & Ratnieks, 1999; Kudô *et al.*, 2007), thus avoiding having their resources usurped by social parasitism or any other kind of opportunistic interaction (Ortolani *et al.*, 2010; Costanzi *et al.*, 2013). This means that each colony has a specific chemical recognition signature familiar to all members of the same colony, thereby avoiding this problem and making the cuticular chemical profile of each individual of the colony vital.

It is clear that the insect's cuticle plays a dual role in that CHCs mediate the transmission of information between individuals and keep the physical structure of the waxy layer on the cuticle intact and impermeable (Gibbs, 1998, 2007; Le Conte & Hefetz, 2008; Chown *et al.*, 2011; Boulay *et al.*, 2017; Chung & Carroll, 2015). Still, little is known about the effect(s) of one function on the other in social insects (Menzel *et al.*, 2018), and no study has evaluated this relationship among social wasps.

*Polybia paulista* is a species of social wasp belonging to the Vespidae family (Richards, 1971), subfamily Polistinae, and tribe Epiponini (Carpenter & Marques, 2001). The species has nests covered with an envelope, with their combs vertically overlapping (Richards, 1971; Carpenter & Marques, 2001). *Polybia paulista* displays a wide geographic distribution in Brazil, being found in the states of São Paulo, Goiás, Mato Grosso, Mato Grosso do Sul, Paraná, and Minas Gerais. In addition to Brazil, the presence of *P. paulista* has already been reported in Paraguay and Argentina (Richards, 1978; Mendonça *et al.*, 2017; Michelutti *et al.*, 2018; Batista *et al.*, 2022). Furthermore, *P. paulista* is an easy-to-collect species and its colonies have a large number of individuals (Kudô *et al.*, 2005), making it an interesting study model. Given its wide distribution, *P. paulista* has been used in studies of characterization and biotechnological application of its venom (Jacomini *et al.*, 2013; Mendonça *et al.*, 2017; Souza *et al.*, 2019), as well as its vulnerability to infection. neonicotinoid insecticide, a compound widely used in Brazilian crops (Batista *et al.*, 2022). The wide geographic distribution of *P. paulista* also exposes colonies to different temperature conditions.

This is the first study to test the hypothesis that the level of aggressiveness presented during induced encounters between workers of *P. paulista* exposed to temperature variation and nestmates who remain under constant temperature will be significantly different than the level of aggressiveness of the encounters between nestmates that remain isolated but under the same temperature. If the hypothesis is confirmed, it is likely because the cuticle of the wasps that were exposed to temperature variation adjusted to these conditions (Michelutti *et al.*, 2018), altering the chemical cues of recognition of nestmates.

## MATERIAL AND METHODS

### Collection and habituation

In February 2017, four colonies of *P. paulista* were collected in the surroundings of the municipality of Dourados, MS, Brazil (22°13'16"S; 54°48'20"W). All colonies were taken from the nesting site at dusk by detaching from the substrate and wrapping them in a plastic bag. In this species, small colonies are expected to have around 305 adult females and five queens. Large colonies can reach up to 6,193 adult females and 24 queens (Kudô *et al.*, 2005). After collection, the wasps of each colony were kept in the laboratory in 20-liter plastic boxes (29.50 × 41.00 × 26.00 cm) under a constant temperature of 24°C and fed with water and honey ad libitum during 24 hours for habituation.

### Effect of temperature variation on the level of recognition of nestmates

After the acclimatization period, five 500-mL plastic containers containing five wasps from the same colony were placed in a BOD (biochemical oxygen demand)

chamber with a programmed temperature ( $24 \pm 2^\circ\text{C}$  and a light-dark cycle of 12:12 h). Each plastic container was drilled in the lid, base, and sides to allow airflow, and all of them were wrapped with red cellophane paper to avoid stressing the wasps. From each colony, another five containers with the same number of wasps were kept out of the BOD in laboratory conditions at a constant temperature ( $24 \pm 2^\circ\text{C}$  with the same light and dark cycle of 12:12 h). The wasps were fed with honey and water throughout the experiment period in microtubes.

The wasps were exposed to an initial temperature of  $15^\circ\text{C}$  for 24 h in the BOD. After 24 h, one plastic container was removed from BOD (Michelutti *et al.*, 2018), and the wasps of this container were paired in induced encounters with a wasp of one of the containers that had been kept outside the BOD at the temperature under laboratory conditions ( $24^\circ\text{C}$ ). For the remaining five plastic containers in the BOD chamber, the temperature was kept at  $20^\circ\text{C}$  for 24 h when the second plastic container was removed from the BOD, and the procedure described above was repeated. Thus, experiments were conducted following this procedure, ranging the temperatures from  $15^\circ\text{C}$  to  $35^\circ\text{C}$  (Fig. 1).

The temperatures of assay were selected as the average of the two main seasons from the previous year (Zavardini, 1992): the dry and cold season with an average of  $20.6 \pm 5.0^\circ\text{C}$  and the minimum average reaching  $14.6 \pm 5.0^\circ\text{C}$  and the wet and hot season with a mean of  $24 \pm 3.3^\circ\text{C}$  and maximum average reaching  $31.1 \pm 3.9^\circ\text{C}$  (Embrapa Agropecuária Oeste from 2016 and 2017). These are temperatures at which wasps may suffer exposure in their natural environment (Michelutti *et al.*, 2018).

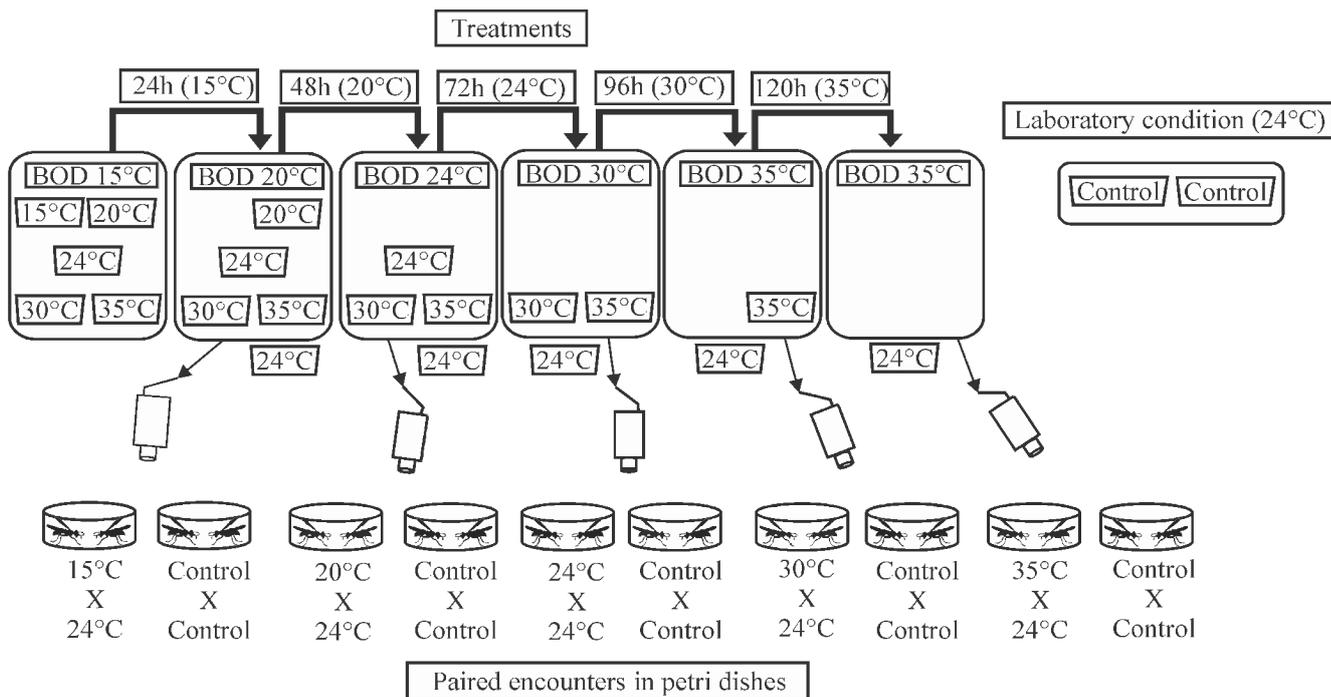
The effect of isolation of wasps was evaluated using ten plastic containers with five wasps from the same colony, which were kept isolated. Induced encounters

between wasps from two different containers from the same colony were held every 24 h. The induced encounters between the wasps of the last two plastic containers were held after 120 h, the same time as the group of wasps that were exposed to temperature variations up to  $35^\circ\text{C}$  kept in the BOD (Fig. 1).

The induced encounters were performed in a Petri dish ( $9.5 \times 2.0\text{ cm}$ ). First, one wasp that was in a plastic container under laboratory conditions ( $24^\circ\text{C}$ ) was placed in the dish, and after 1 min, a wasp from BOD, which had been subject to temperature variation, was placed in the same Petri dish. During encounters, the interactions between the two wasps were recorded for 15 min without interruption with the aid of a Sony HDR C220 camera. A blinded observer then analyzed the recordings. The observer then took notes regarding the type and number of times each behavior was executed by each wasp.

To evaluate the level of intercolonial aggressiveness and compare it with the levels of aggressiveness displayed between nestmate wasps in the experimental encounters, 20 wasps per colony ( $n = 3$  colonies) were used in a total of 30 paired encounters (Colony 1  $\times$  Colony 2; Colony 1  $\times$  Colony 3; Colony 2  $\times$  Colony 3). We used the same method to evaluate the level of aggressiveness between the two wasps at each encounter by counting the number of times, *i.e.*, absolute frequency, that three behavioral acts occurred: stationary alarm, biting, and stinging, all considered agonistic behaviors by O'Donnell (2003) and Togni & Giannotti (2007).

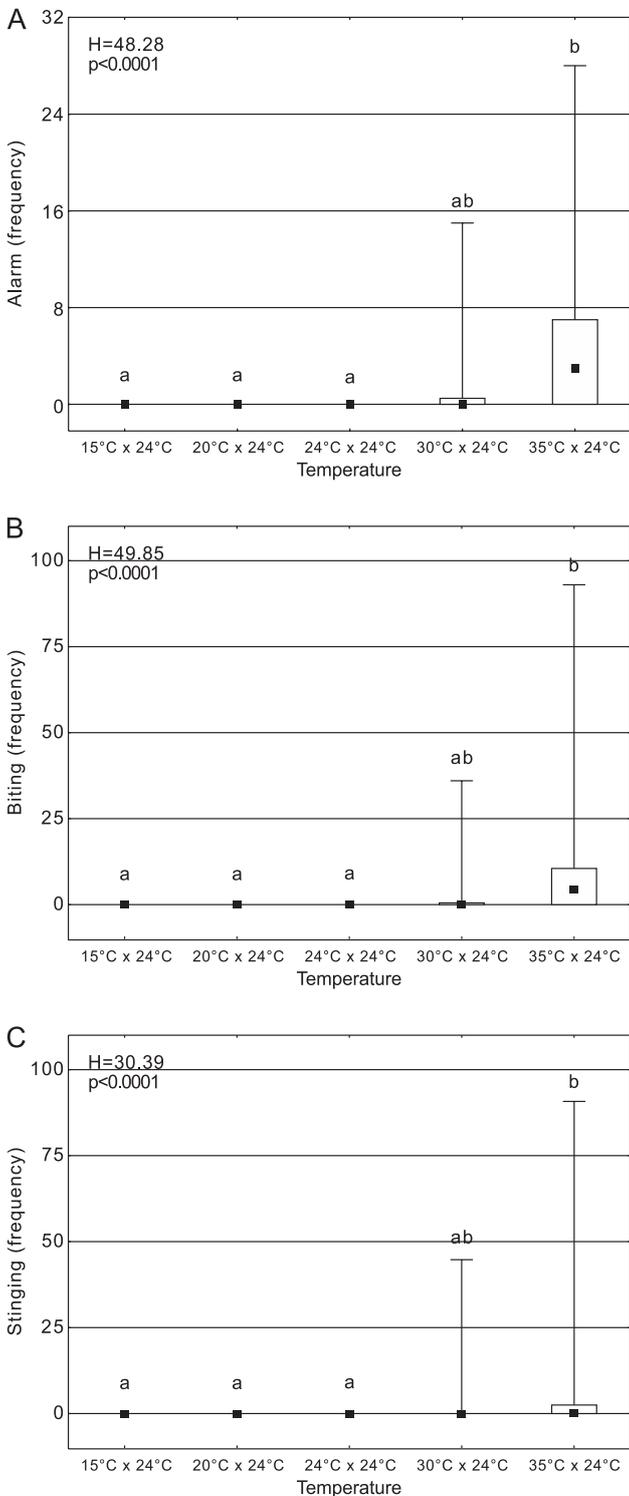
To assess the level of aggressiveness during the induced encounters, a 3-point scale (adapted from Valadares *et al.*, 2015) was used: 1 = antennation; 2 = Bite, and 3 = attack. Subsequently, the aggression index (AI) was calculated, and the scores range from 0 (where there



**Figure 1.** Graphic representation of the experimental design. Containers inside the BOD represent wasps subjected to different temperatures used to evaluate the effect of temperature on behavior. Containers outside the BOD ( $24^\circ\text{C}$ ) represent wasps kept under laboratory conditions and used in the paired encounters with experimental wasps. Control containers represent wasps used in the experiments to evaluate isolation's effect on nestmates' recognition.

was no aggressive interaction 2 and 3) to 1.0 (highest AI score). For the calculation of the index, we use the following formula: A.I. = number of interactions 2 and 3/total number of all interactions.

According to O'Donnell (2003) and Togni & Giannotti (2007), alarm involves pumping the gaster, contracting it



**Figure 2.** Median (square), quartile range (box) and total range (vertical line) of the frequency of antagonistic behaviors – (A) Alarm, (B) Biting, (C) Stinging – in each treatment (15°C × 24°C, 20°C × 24°C, 24°C × 24°C, 30°C × 24°C, 35°C × 24°C) and the results of the Kruskal-Wallis test (n = 100). Different lowercase letters (a, b) indicate significant differences between treatments (p < 0.05).

repeatedly and/or repeating the act of lifting and lowering wings. Wasps perform biting by moving fast toward another wasp with the jaw open and closing it when they touch. Stinging behavior occurs when the end of the gaster is bent toward another wasp, and the stinger is inserted.

The same procedure was repeated for induced encounters between wasps that tested the effect of isolation and between wasps of different colonies. No wasp has been marked with paint or any other type of marking to prevent interference with the results.

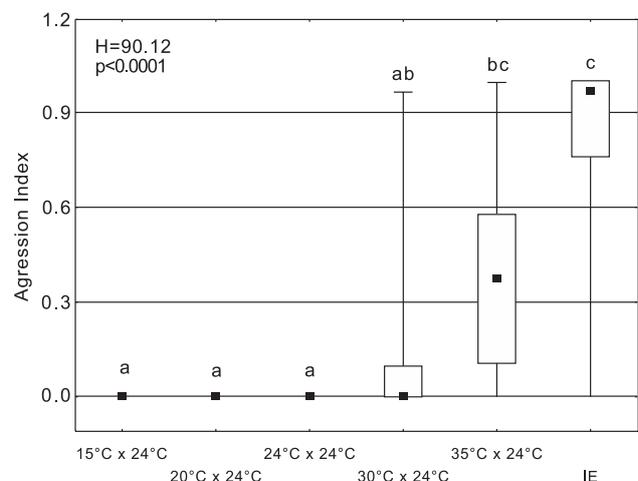
### Statistical analyses

In order to evaluate any significant differences in the frequency of each antagonistic behavior (stationary alarm, biting and stinging) among the treatments (15°C × 24°C, 20°C × 24°C, 24°C × 24°C, 30°C × 24°C, and 35°C × 24°C), we applied a Kruskal-Wallis test ( $\alpha = 0.05$ ), using Statistica 14 (TIBCO, 2020). When necessary, we applied the *a posteriori* test to compare treatments. The results were presented graphically, using different lowercase letters to indicate significant differences between treatments.

Next, we used the same statistical procedure to compare the Aggression Index between treatments (15°C × 24°C, 20°C × 24°C, 24°C × 24°C, 30°C × 24°C, and 35°C × 24°C), including the data obtained in the encounters between wasps from different colonies.

### RESULTS

A total of 25 hours of interactions between the wasps of different groups were recorded. No manifestation of agonistic behaviors (stationary alarm, biting and stinging) was observed among individuals that remained iso-



**Figure 3.** Median (square), quartile range (box) and total range (vertical line) of the Aggression Index in each treatment (15°C × 24°C, 20°C × 24°C, 24°C × 24°C, 30°C × 24°C, 35°C × 24°C) and between individuals from different colonies Intercolonial encounters (IE). The results of the Kruskal-Wallis test (n = 128) are also presented. Different lowercase letters (a, b, c) indicate significant differences between treatments (p < 0.05).

lated for 24 hours up to 120 hours (Fig. 2). We also did not observe any agonistic act during the meetings between wasps that experienced the temperature variation from 15°C to 24°C with those that remained under laboratory conditions (Fig. 2). On the other hand, agonistic acts were performed during the meetings between wasps that remained in the BOD up to 30°C and 35°C and those that remained under laboratory conditions (Fig. 2).

Figure 3 shows that only the encounters between 30°C × 24°C and 35°C × 24°C and between wasps from different colonies presented some level of aggressiveness. The levels of aggressiveness between 30°C × 24°C were statistically lower than those presented by wasps of different colonies. However, there was no significant difference between the levels of aggressiveness shown during the encounters between 35°C × 24°C and wasps from different colonies (Fig. 3).

## DISCUSSION

According to our results, aggressive behaviors occurred between nestmates that remained under different temperatures, in particular, those that remained laboratory conditions and those that were kept in BOD up to the temperatures of 30°C and 35°C (Figs. 2 and 3). During these encounters, wasps performed alarm behavior, bites, and stings not seen during encounters between wasps kept in isolation for the same period. The lack of aggressive behavior under isolated conditions indicates that isolation had no effect on chemical recognition signature. These results suggest that temperature differences may have caused some change in the cues that allow recognition between nestmates (Figs. 2 and 3).

As previously demonstrated by other authors, the separation of nestmate wasps might cause changes in the CHC profile owing to the presence or absence of the queen that performs pheromonal control, acting directly on the physiological development and behavior of workers (Holman *et al.*, 2010; Van Oystaeyen *et al.*, 2014; Oi *et al.*, 2015). In addition, separated nestmates may fail to exchange chemical compounds during social interactions, such as trophallaxis or social cleansing behaviors (Blomquist & Bagnères, 2010). This may also cause some kind of recognition impairment when they meet again. Our experimental design does not explore these issues since they are outside the scope of the present hypothesis. However, we note again that isolation experiments did not reveal any aggressive behavior during induced encounters.

What also helps to reinforce our hypothesis is that, in addition to nestmates usually do not present any kind of aggressiveness between them, even when isolated, wasps from different colonies, on the other hand, are always aggressive to each other, which demonstrates that this species uses chemical cues to recognize nestmates as already described for other species as in the studies by Dani *et al.* (2001), Tannure-Nascimento *et al.* (2007) and Murakami *et al.* (2015). Therefore, nestmates who remained in different temperature conditions probably

exhibited aggressiveness due to some change in the recognition cues when they were subjected to these conditions (Michelutti *et al.*, 2018).

During all induced encounters, as wasps approached one another, the first behavioral act performed was antennation. Notably, antennation between wasps exposed to temperatures of 30°C and 35°C with those held under laboratory conditions (24°C) resulted in aggressive behavior. Both wasps exposed to 30°C and 35°C and those that remained at 24°C under laboratory conditions performed aggressive behaviors, suggesting that aggression was not caused by stress resulting from experimental manipulation or BOD conditions alone.

The present work tests the hypothesis that workers of the wasp *Polybia paulista* may change their CHC profile along temperature variation (Michelutti *et al.*, 2018) and that such change will affect their capacity for intraspecific recognition. Therefore, based on our results, it is likely that recognition impairment and aggression were, in large part, caused by changes in CHC profile relative to variations in temperature and the attempt to adjust to these conditions accordingly. Indeed, we used the same study design, wasp species and temperatures as those of Michelutti *et al.* (2018) who reported that the cuticle compounds of wasps are adjusted according to temperature variation.

In the study with the cockroach *Periplaneta americana* in Cambridge, Ramsay (1935), and Wigglesworth (1945) in Londres with *Blattella germanica*, detected phase change in the cuticle layer and a sudden increase in the rate of evaporation through the cuticle when these insects were subjected to temperature variation, especially when exposed to temperatures above 30°C. Thus, it appears that phenotypic plasticity is permissive for insects responding to changes in the environment, such as temperature (Menzel *et al.*, 2018; Otte *et al.*, 2018). However, taking into account the dual function of hydrocarbons, *i.e.*, communicating and waterproofing (Gibbs 1998, 2007; Le Conte & Hefetz, 2008; Chown *et al.*, 2011; Chung & Carroll, 2015; Boulay *et al.*, 2017; Menzel *et al.*, 2017), the adjustment of one function can have a negative effect on the other, potentially leading to a high cost in social stability of the colony.

Wasps use their ability to differentiate between nestmates and non-nestmates to show greater aggression toward non-nestmates (Lorenzi *et al.*, 1997; Starks *et al.*, 1998; Gamboa, 2004). Indeed, it has already been demonstrated that *P. paulista* has a well-developed nestmate recognition system and that these wasps behave aggressively in contact with non-nestmates, irrespective of the distance between their colonies (Kudô *et al.*, 2007). Therefore, the level of aggression found during interactions between nestmates is not expected, directly pointing to temperature as the determinant of these results.

At the same time, we cannot ignore the stress factor imposed by the study design itself, noting the temperature ranges from 30°C to 35°C and time in the BOD chamber up to 120 h. These stresses could have also led to aggression during the induced encounters. However, the study by Michelutti *et al.* (2018) indicates that the cu-

ticular chemical profile of wasps shows the greatest variation in these same temperature ranges. Furthermore, to reinforce our hypotheses, wasps that remained isolated under constant laboratory conditions (24°C), for the same period, do not demonstrate any level of aggressiveness between them.

Wasps use CHCs as recognition cues between nestmates (Singer & Espelie, 1992, 1996; Lorenzi *et al.*, 1997; Cervo *et al.*, 2002). We know that changes of cuticular profile of *P. paulista* correlate with temperature variation (Michelutti *et al.*, 2018). Although changes in the composition of CHCs allow wasps to readjust to different temperature conditions, our study shows that changes in the composition of compounds can be striking at temperatures above 30°C, so much so that recognition impairment between nestmates can occur, which may cause, in many cases, an increase in aggression between them. Thus, in addition to helping promote the waterproofing of the cuticle, these compounds also serve as cues for the recognition of nestmates.

In the study by Michelutti *et al.* (2018), the major compounds heptacosane and nonacosane more than doubled their percentage in the cuticle of workers of *P. paulista* at these same temperature ranges and this same study design. Corroborating this hypothesis, other studies with *Polistes biglumis bimaculatus* (Lorenzi *et al.*, 1997) and *Mischocyttarus cassununga* (Murakami *et al.*, 2015) reported that heptacosane and nonacosane, both linear alkanes, may be important for the recognition of nestmates in these species. In addition, our results show that the isolation of wasps as a putative determinant of aggressive behavior, as already mentioned, did not cause any difficulty in the recognition of nestmates, reinforcing our overall results. Studies, such as those of Michelutti *et al.* (2018) with *P. paulista* and *Polybia ignobilis* workers and Wagner *et al.* (2001) with the ant *Pogonomyrmex barbatus*, have demonstrated that the cuticle of these species, especially under relatively higher temperatures, undergoes an increase in the percentage of linear alkanes. According to Gibbs (1998), Hefetz (2007), Wagner *et al.* (2001) and Chung & Carroll (2015), increasing the percentage of this class of compounds is important to avoid desiccation in insects and probably occurs to meet the need for waterproofing of the wasp cuticle under high temperatures.

## CONCLUSION

Supporting our hypothesis, encounters between wasps that suffered temperature variation when induced to interact with nestmates not subjected to these conditions presented levels of recognition impairment different from the normal pattern, probably as a function of CHC composition adjustment, as observed by Michelutti *et al.* (2018).

**AUTHORS' CONTRIBUTIONS:** SELJ: Formal analysis; WFAJ: Project administration; CALC, KBM, WFAJ: Conceptualization; CALC, KBM, NRB, SELJ, WFAJ: Methodology; Writing – review & editing; KBM, NRB:

Investigation; KBM, NRB, WFAJ: Writing – original draft, Visualization; KBM, NRB, SELJ: Data Curation; CALC, WFAJ: Resources, Supervision. All authors actively participated in the discussion of the results; they reviewed and approved the final version of the paper.

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