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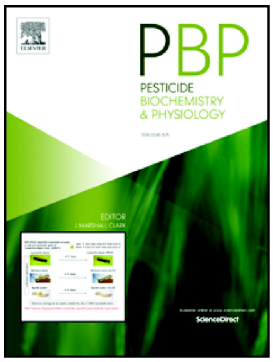

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Samira Veiga Ravaiano, Wagner Faria Barbosa, Hudson Vaner Ventura Tomé, Lúcio Antônio de Oliveira Campos, Gustavo Ferreira Martins

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Acute and oral exposure to imidacloprid does not affect the number of circulating hemocytes in the stingless bee *Melipona quadrifasciata* post immune challenge

Samira Veiga Ravaiano[a], Wagner Faria Barbosa[a], Hudson Vaner Ventura Tomé[b], Lúcio Antônio de Oliveira Campos[c], Gustavo Ferreira Martins[c,*] gmartins@ufv.br

[a]Departamento de Entomologia, Universidade Federal de Viçosa, 36570-900, Viçosa, MG, Brazil

[b]EAG Laboratories, 13709 Progress Boulevard no. 24, Suite S163, Alachua, FL, 32615, USA

[c]Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570-900, Viçosa, MG, Brazil

[*]Corresponding author at: Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV), Viçosa, MG 36570-900, Brazil.

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Abstract

In the present work, the effects of the neonicotinoid imidacloprid formulation on the total hemocyte counts (THC) and differential hemocyte counts (DHC) were investigated in foraging workers of the stingless bee *Melipona quadrifasciata* under (or not) the challenge with the bacteria *Escherichia coli*. The THC was not altered with the insecticide exposure and/or bacterial infection. However, the DHC of the bees changed with the imidacloprid exposure and/or bacterial infection. The number of prohemocytes (stem cells) increased in bees exposed to imidacloprid, but it did not change after the bacterial infection. The number of plasmatocytes (phagocytic cells) increased in imidacloprid-exposed and uninfected bees and decreased in infected bees regardless of the exposure to imidacloprid. On the other hand, there was a reduction of granulocytes, the most active immune cells, after imidacloprid exposure and an increase of granulocytes after the infection. Previous studies have shown that the neonicotinoids exposure can impair the immune system of bees. Our findings showed that the relative number of granulocytes declined with imidacloprid exposure, but the overall capacity of hemocyte responses in terms of total numbers after bacterial infection persisted even after the insecticide exposure.

Keywords: cellular response, neonicotinoid, insect immune system, wild bee

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1 Introduction

The recurrent debates over pollinator declines almost always end up on bee species from temperate regions [1,2]. This is not surprising as the popularity of these bee species is high even in regions where they are exotic and show less expressiveness in migratory beekeeping or greenhouse pollination services, just as with the honey bee *Apis mellifera* [3]. On the other hand, little progress has been made yet in expanding ecotoxicological studies and risk assessments on bee species more representative in sustaining agricultural and natural ecosystems on their native landscapes, such as in the tropics [1,2,4].

In pantropical areas, a diverse group of stingless bee species performs a pivotal pollination service for native and cultivated plants and such species therefore have an irreplaceable ecological and agronomic function [1,2,5]. This is also the case with the eusocial stingless bee species of genus *Melipona* (Hymenoptera: Apidae: Meliponini), which are important pollinators of plants such as tomatoes [6,7], bell peppers [8], and eggplants [9] that have their productivity and fruit quality enhanced by the anther vibration caused by stingless bee workers during pollination [10–13].

Pollination efficacy may drop with decline in the bee populations, which is usually associated with multiple factors including habitat fragmentation, climate change, decreasing resource diversity, pathogen attacks, and use of pesticides [2,14] like neonicotinoids [15]. Neonicotinoid insecticides are agonists of nicotinic acetylcholine receptors (nAChR) and can lead to constant activation of cholinergic synapses, hyperexcitation, and death [16]. Neonicotinoid are absorbed by plants and can accumulate in the leaves, pollen, and nectar, thereby enhancing the risk of exposure to non-target insects including bees [17]. In addition, neonicotinoids demonstrated to disrupt immune reactions in insects [18–20].

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Immune responses in insects depend in part on hemocyte activities, which comprise phagocytosis, nodulation, and encapsulation [21,22]. Among hemocytes, granulocytes and plasmatocytes are the most active cells [21,23], and prohemocytes are stem cells that give rise to other hemocytes [23,24]. These cells can be differentiated by their morphology: granulocytes are characterized by being round or slightly oval, as well as their nucleus, which occupies a central position; plasmatocytes are polymorphic cells and can be found as moon, fusiform or oval shapes, with oval and central nucleus; lastly, prohemocytes are the smallest hemocytes found in the hemolymph showing round or slightly oval shapes, with proportionally large nuclei occupying almost the entire volume of the cell and limiting the cytoplasm to a thin peripheral band [25,26]. Although pesticides weaken the immune response in bees, the mechanisms underlying the responses of specific hemocyte populations to pesticide exposure are still unknown [18].

In the present study, the impact of a frequently-used neonicotinoid (imidacloprid) on hemocyte populations was assessed in bacteria-infected foragers of the neotropical stingless bee *Melipona quadrifasciata*, through total and differential hemocyte counts (THC and DHC, respectively). Surprisingly, the overall capacity of hemocyte responses in *M. quadrifasciata* persisted even after insecticide exposure, which has been controversially demonstrated in other bee species such as *A. mellifera* [18]. The peculiarities of this result are therefore discussed.

2 Material and Methods

2.1 Insects and bacteria

The bees were placed in groups per colony in wooden cages (30 × 30 × 30 cm) and starved for 1 h under complete darkness at room temperature. In the experiments, the average of

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data from bees in each different colony was used as a biological replicate (i.e., four replicates were used). The bacteria *Escherichia coli* K-12 (1.3×10^7 colony forming units; CFU/mL) were cultivated in BD Brain Heart Infusion Agar (BHI Agar, Becton Dickinson Company, Heidelberg, Germany) 24 h prior to their injection in bees.

2.2 Acute insecticide exposure and bacterial infection

The commercial formulation Evidence WG (*N*-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide; 700 g of imidacloprid (a.i.)/kg, water dispersible granules; Bayer CropScience, São Paulo, SP, Brazil) was diluted in 50% sucrose in distilled water at a concentration of 292 ng a.i./mL, based on a very low toxicity dose estimated for *M. quadrifasciata* (LD_1 = 2.92 ng a.i./bee) [27] and offered to the bees. Workers starved for 1 h were individualized in cylindrical glass tubes (15 cm length and 1.5 cm diameter) sealed with parafilm and provided with 10 µL of imidacloprid-contaminated sucrose solution [27]. Other individuals received uncontaminated sucrose solution as a control. Bees that completely depleted the honey solution up to 5 min were transferred to 200 mL plastic pots with an uncontaminated sucrose solution provided *ad libitum* via a drilled microtube-made feeder and were maintained for 24 h at 28°C and 75% RH under complete darkness.

The insects were cold anesthetized (4°C) and inoculated with 10 µL of LB medium containing *E. coli* (1.3×10^5 CFU/bee) into the 1st tergite using an insulin syringe (BD Ultra-Fine, 0.25-mm gauge needle, Uniqmed, São Paulo, SP, Brazil). Another group of insecticide-uncontaminated bees was inoculated with sterilized BHI Agar, as an additional treatment group.

The experiment was carried out based on a completely randomized design under a factorial scheme (2^2 + 1), which resulted in five treatments. The factors were insecticide

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exposure and bacterial infection, encompassing two levels (presence or absence) and the individuals injected with LB medium comprised the additional treatment.

2.3 Differential and total hemocyte counts

One hour after bacterial inoculation, bees were anesthetized and inoculated with 20 µL of an anticoagulant solution (0.098 M NaOH, 0.145 M NaCl, 0.017 M EDTA and 0.041 M citric acid; pH 4.5) into their dorsal pleura between the 1st and 2nd abdominal tergites. The "hemolymph" (hemolymph + anticoagulant solution) was collected using a micropipette with a siliconized tip from the same wound created after the injections and expelled by careful manual compression of the abdomen [26].

For THC, 10 µL of hemolymph from a single individual was collected and dispensed directly (without dilution) into an improved Neubauer chamber. The count was carried out under a microscope (400× magnification) [26]. The mean value of two to four individuals from each colony (i.e. the replicate) was used per treatment (i.e. ~12 bees per treatment), and in total, ~60 individuals were used.

For DHC, the maximum volume of hemolymph expelled from each bee was collected and dispensed on glass slides, allowed to adhere for at least 20 min at room temperature. Smears were fixed in methanol for 10 min and stained with Giemsa solution [25]. Cells were photographed using a digital camera Axio Cam ERc5s coupled to the microscope (Primo Star, Zeiss) and identified. Three hundred cells were counted per individual to estimate the prohemocyte, granulocyte, and plasmatocyte numbers, and the average value of two individuals from each colony (i.e. the replicate) was used per treatment (i.e. eight bees per treatment), totalizing 40 individuals used. The three main

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circulating hemocytes of *M. quadrifasciata*, i.e. prohemoctes, granulocytes, and plasmatocytes, were identified according to their morphology as described elsewhere [25,26,28].

2.4 Statistical analyses

Data were tested for normality and homoscedasticity and subjected to variance analyses and post-hoc Tukey's test using the package [ExpDes] of the software R (R 2016, vs. 3.3.2) [28], considering the factorial scheme ($2^2 + 1$).

3 Results

3.1 Total hemocyte counts (THC)

Neither insecticide exposure ($F_{1,15} = 0.02$; $p = 0.89$; Supplementary tables I and II) nor bacterial infection ($F_{1,15} = 0.42$; $p = 0.53$; Supplementary tables I and II) affected the THC of *M. quadrifasciata* foragers. The overall mean was 284.7 ± 41.3 (× 1000) cells irrespective of exposure to imidacloprid or bacterial infection. Nevertheless, the THC of bees treated with only sterilized LB (i.e. bees from the additional treatment) was significantly higher than that in bees subjected to imidacloprid contamination and bacterial infection together (namely, factorial treatment) ($F_{1,15} = 13.73$, $p = 0.002$; Supplementary tables I and II).

3.2 Differential hemocyte counts (DHC)

Exposure to imidacloprid interfered with the DHC considering the prohemocytes, plasmatocytes, or granulocytes regardless of infection with *E. coli*. Such interference occurred either by interaction or non-interaction between insecticide exposure and bacterial

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infection (Figures 1 and 2). In addition, the DHC of individuals injected with LB alone (i.e. the additional treatment) was similar ($p > 0.05$) to that in individuals exposed to imidacloprid and/or infected with bacteria (i.e. factorial treatment) for any of the cells (Table I).

Imidacloprid exposure increased the relative number of prohemocytes compared to that in non-exposed bees ($F_{1,15} = 21.60$; $p < 0.001$; Figure 1a; Supplementary tables III and IV). However, no effect of bacterial infection was detected in these cells ($F_{1,15} = 1.30$; $p = 0.27$; Figure 1b; Supplementary tables III and VI).

There was significant interaction between insecticide exposure and bacterial infection ($F_{1,15} = 6.66$; $p < 0.02$; Figure 2c, Supplementary tables V and VI) considering the number of plasmatocytes. The number of these cells was higher in imidacloprid-exposed bees (89.38 ± 7.31) than in unexposed bees (47.63 ± 3.31) with no bacterial inoculation ($F_{1,15} = 29.35$; $p < 0.001$; Figure 2c). On the other hand, the number of plasmatocytes was similar between imidacloprid-exposed (43.75 ± 8.18) and unexposed bees (30.13 ± 3.54), which were both infected with *E. coli* ($F_{1,15} = 3.13$; $p = 0.10$; Figure 2c). Bacterial infection also reduced the number of plasmatocytes in both imidacloprid-exposed ($F_{1,15} = 35.05$; $p < 0.001$; Figure 2c) and unexposed bees ($F_{1,15} = 5.16$; $p = 0.04$; Figure 2c).

There was no significant interaction between insecticide exposure and bacterial infection ($F_{1,15} = 3.93$; $p = 0.07$, Supplementary tables VII and VIII) with respect to granulocytes. Imidacloprid reduced the number of granulocytes (219.50 ± 9.83) compared to that in unexposed bees (253.62 ± 3.92) ($F_{1,15} = 30.48$; $p < 0.001$; Supplementary tables VII and VIII; Figure 3a), whereas infection with *E. coli* increased the number of these cells (251.31 ± 6.36) compared to that in uninfected bees (221.81 ± 9.62) ($F_{1,15} = 22.78$; $p = 0.002$; Supplementary tables VII and VIII; Figure 3B).

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4 Discussion

Pesticides, mainly neonicotinoids, have been pointed out as enhancers of bee declines globally [1,2]. The concern about pesticidal impact on pollinators has been supported by several (mainly laboratorial) evidences of toxicological impairment in bees, including effects that are not associated with the mode of action of the tested compounds. These side effects may be observed with neurotoxic insecticides such as neonicotinoids as disturbances in immune reactions for instance [18–20]. However, the prevalence of toxicological studies and risk assessments with the honey bee *A. mellifera*, whose popularity as a surrogate pollinator remains unchanged, has led to a neglected view of other pollinators such as stingless bees, which are assumed to be more representative in their native landscapes [1,2]. In the present study, we assessed the cellular response of the innate immune system in *M. quadrifasciata*, an important pollinator of native and agronomic plants in the Neotropical Region [6,7], exposed to a sublethal dose of the neonicotinoid. imidacloprid and post-challenged with bacteria.

The THC of *M. quadrifasciata* foragers was not affected by insecticide exposure or bacterial infection. In contrast, exposure of *A. mellifera* nurses to neonicotinoids, including imidacloprid, leads to reduction in the THC [18]. This suggests that the effects of imidacloprid exposure depend on the species, exposure method, and the age of the bees studied. Regardless of the unchanged THC upon both imidacloprid exposure and bacterial infection, injection of BHI Agar resulted in increased THC, which has also been described elsewhere [30].

The DHC of stingless bee foragers injected with LB medium alone (as an additional control treatment) resembled that of individuals regardless of imidacloprid exposure and/or bacterial infection. On the other hand, imidacloprid exposure enhanced the number of

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prohemocytes to almost twice that in non-exposed bees, but no effect of bacterial infection was detected in these cells. An increase in prohemocytes was also reported for foragers of the honey bee *Apis dorsata* exposed to imidacloprid [19]. This increase in prohemocytes supposedly occurs to cater the demand for the formation of other hemocytes in exposed individuals [24].

Imidacloprid exposure also increased the number of plasmatocytes, but only in non-infected bees. The number of plasmatocytes was also increased under imidacloprid exposure in *A. dorsata* simultaneously with the proliferation of prohemocytes [19]. Such an increase seems to be linked to the hormesis phenomenon (i.e. an advantageous consequence of low doses of compounds that are toxic at high doses) [31]. In contrast, the reduction of plasmatocytes caused by bacterial infection in imidacloprid-exposed or non-exposed individuals may occur by the attachment of these cells to the body wall or their recruitment for nodule formation as a response to the infection [32].

Among the three circulating hemocytes in *M. quadrifasciata*, granulocytes were the most numerous, comprising 79.3% of the total. For granulocytes, the impact of both insecticide exposure and bacterial infection was inverse. Imidacloprid exposure leads to a reduction in the number of granulocytes, which does not seem to be directly linked to the neurotoxic effect of this compound [16,17], but may be attributed to a secondary effect [18] or to other components of the tested formulation [33]. In contrast, infection with *E. coli* leads to an increase in the number of granulocytes, which is in accordance with reports on other insects [34,35].

Overall, acute/oral exposure of foragers of the eusocial bee *M. quadrifasciata* to a sublethal dose of imidacloprid *in vitro* leads to specific changes in the population of circulating hemocytes. Exposed bees demonstrated an increase in the number of

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prohemocytes and plasmatocytes (the latter only in non-infected bees). In contrast, imidacloprid exposure caused a reduction of granulocytes, which constitute the most numerous immune cells. However, this suppression did not interfere with the number of granulocytes when the bees were challenged with bacteria.

Neonicotinoid exposure was previously shown to reduce the populations of hemocytes interfering with the humoral response of honey bees [18], which could render them more susceptible to pathogens. In addition, favoring of viral pathogenicity in honeybees has also been demonstrated by reduction of their immune defenses via impairment of insect NF-κB immune signaling [36]. However, the link between pesticides and the ability to respond immunologically against pathogens may vary among species and is still not well clarified in bees. Our results indicate the need for more studies to understand how neonicotinoid formulations interfere with cellular and humoral responses and with the susceptibility of bees to pathogens. Despite the slight impairment of hemocyte counts in *M. quadrifasciata* by imidacloprid, sublethal doses of imidacloprid affect other physiological and behavioral characteristics of this stingless bee such as respiration, brain development, and locomotor behavior [27,37], which may possibly imply a threat for the conservation of this native bee species.

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Table I. Differential hemocyte counts (± standard error [SE]) in *Melipona quadrifasciata* foraging workers subjected to additional (i.e. exposure to uncontaminated sucrose solution and inoculation with sterile Luria Bertani medium) and factorial (i.e. combination of bees exposed or not exposed to imidacloprid and infected or not infected with *Escherichia coli*) treatments. *p* values greater than 0.05 indicate absence of significant difference, as determined by Tukey's test.

Hemocytes	DHC (± standard error [SE])		$F_{1,15}$	*p* value
	Additional	Factorial		
Prohemocytes	12.1 ± 1.8	10.8 ± 1.1	0.68	0.42
Plasmatocytes	44.3 ± 2.1	52.7 ± 6.3	1.93	0.18
Granulocytes	243.6 ± 2.9	236.6 ± 6.7	1.04	0.32

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Figure 1. Relative number of prohemocytes and plasmatocytes in the foraging workers of *Melipona quadrifasciata*. Bees were subjected to imidacloprid exposure (a) and *Escherichia coli* infection (b). Only the effect of insecticide exposure was detected, which indicated a significant increase in the number of prohemocytes in imidacloprid-treated bees compared to the non-exposed bees. (c) Imidacloprid exposure and bacterial infection together cause a change in the relative number of plasmatocytes. Imidacloprid exposure increased the number of cells in non-infected bees, while the number of these cells remained unchanged in infected individuals. The infection decreased the cell number, independently of imidacloprid exposure. Different letters represent significant difference, as determined by Tukey's test ($p < 0.05$), between insecticide exposure in the presence or absence of bacterial infection. Asterisks in (c) represent a significant difference ($p < 0.05$) in the extent of bacterial infection in the imidacloprid-treated and untreated groups.

Figure 2. Relative number of granulocytes in the foraging workers of *Melipona quadrifasciata* subjected to imidacloprid exposure and *Escherichia coli* infection. Insecticide exposure (a) and bacterial infection (b) independently affected the relative number of cells. Different letters represent significant difference, as determined by Tukey's test ($p < 0.05$).

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Highlights

- The effects of imidacloprid on the hemocyte were investigated in stingless bees.
- The total number of hemocytes was not altered with the insecticide exposure.
- The number of prohemocytes increased after the imidacloprid exposure.
- The number of granulocytes declined with imidacloprid exposure.
- The overall capacity of hemocyte responses persisted even after the exposure.

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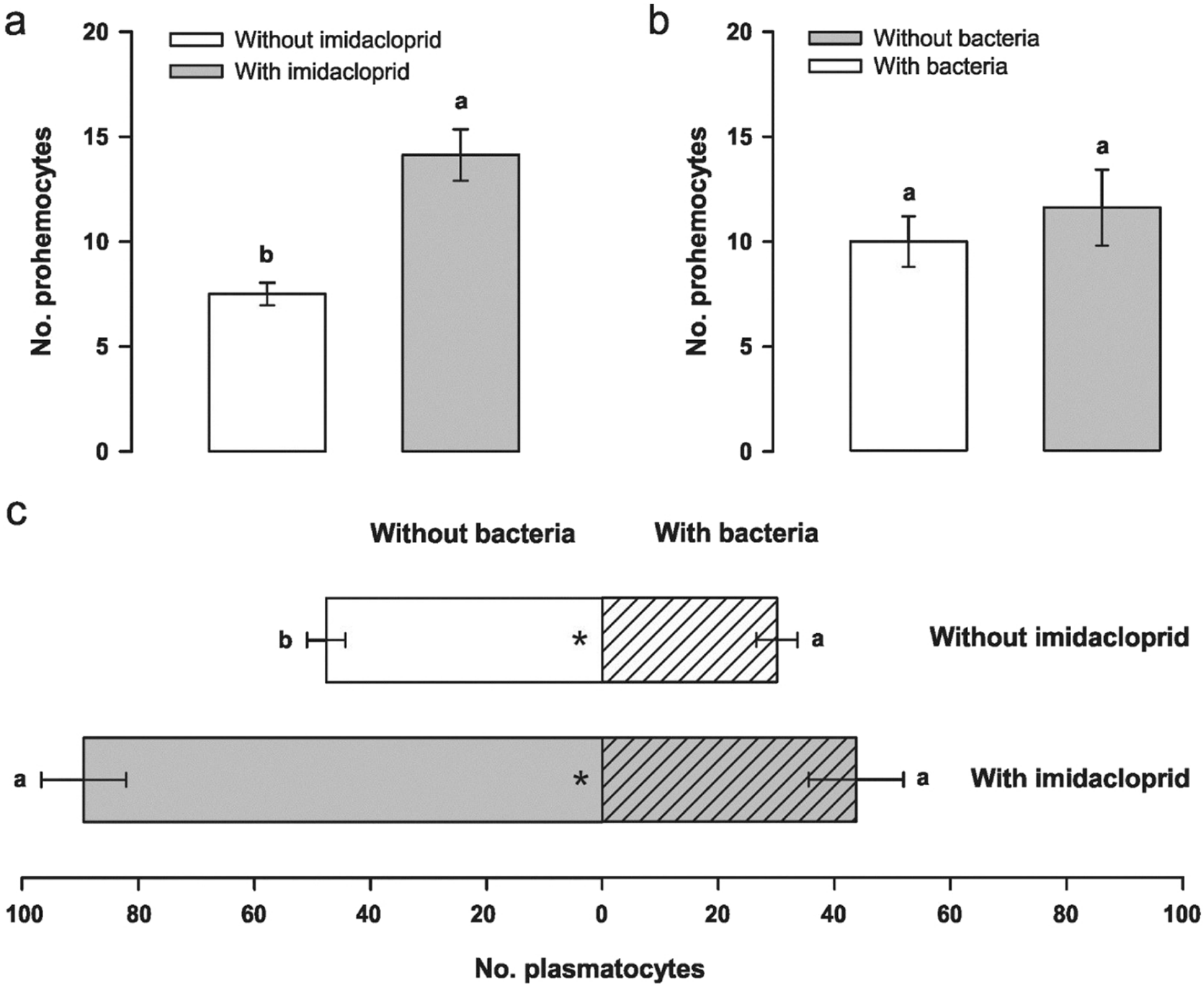
a
Without imidacloprid
With imidacloprid
No. prohemocytes
20
15
10
5
0
b
a
b
Without bacteria
With bacteria
No. prohemocytes
20
15
10
5
0
a
a
c
Without bacteria
With bacteria
b
*
a
Without imidacloprid
a
*
a
With imidacloprid
100
80
60
40
20
0
20
40
60
80
100
No. plasmatocytes

Figure 1

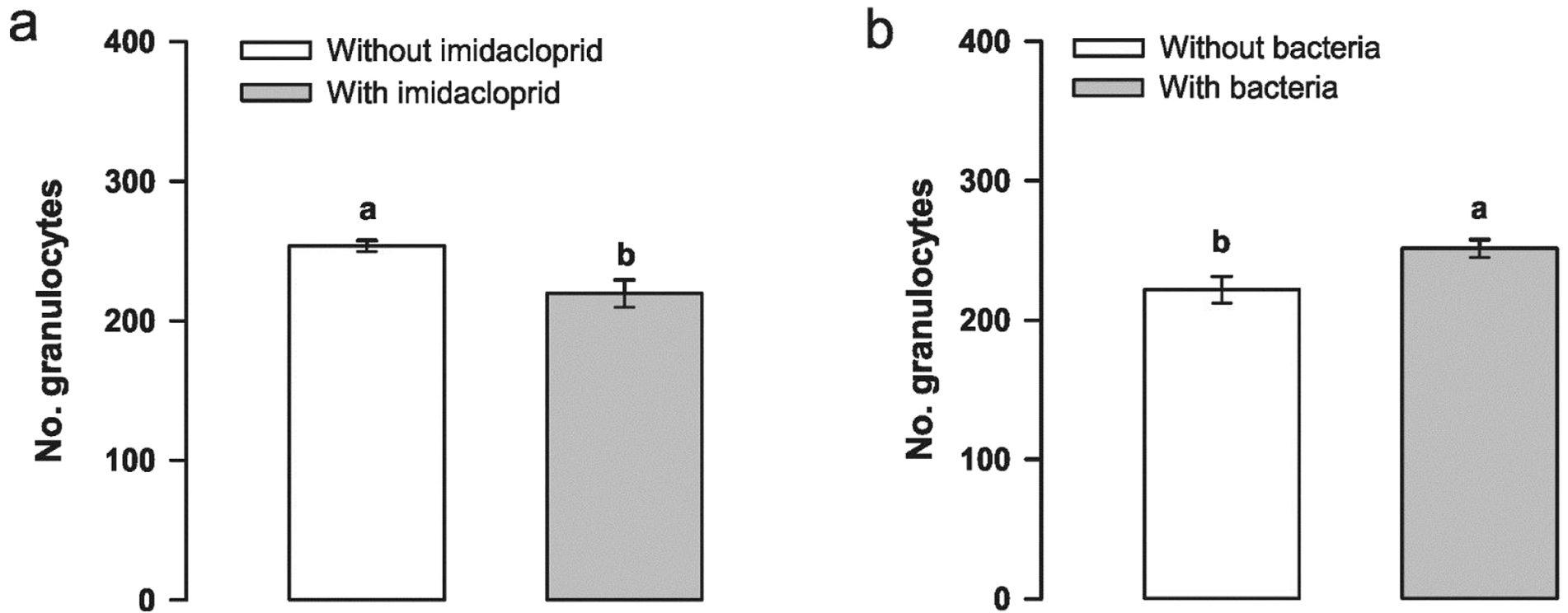
a
400
300
200
100
0
No. granulocytes
Without imidacloprid
With imidacloprid
a
b
b
Without bacteria
With bacteria
a

Figure 2