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Safe sex: ant defense does not interfere with pollination in passion flowers

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ABSTRACT

Ant defense against floral enemies incurs a two-fold ant-pollinator conflict, both via pollinator deterrence and nectar or pollen collection by non-pollinating protective ants. Some ant-plants have physical barriers whereas others produce ant-repellent chemicals to avoid ant visitation to flowers and subsequent pollination interference. *Passiflora coccinea* is a hummingbird-pollinated myrmecophilous plant in which floral enemy repellence occurs without limiting ant access to open flowers. To test the hypothesis that ant activity is restricted within flowers to prevent contact with anthers, we compared ant defense response between reproductive (anthers and stigmas) and non-reproductive (bracts, corona and perianth) floral structures by combining an observational survey with an experimental approach. A few insect species were found to visit flowers without providing pollination service, mostly pollen-collecting bees and nectar-thieving butterflies landing on petals. Ants always attacked floral visitors that landed on non-reproductive structures, but they never attacked insects visiting reproductive structures as ants never accessed anthers. Our results suggest that the differential ant defense response is an adaptative process to prevent ant-pollinator conflict. The eventual mechanism that regulates this process could be closely linked to the corona of filaments that protects nectar chambers, simultaneously restricting ant access to nectar and pollen.

Keywords: ant-pollinator conflict, ant protection, corona, extrafloral nectaries, extranuptial nectaries, floral enemies, flower reproductive structures, nectar thieves, *Passiflora coccinea*, pollination

Introduction

Animal-pollinated plants have to deal with a diverse assemblage of illegitimate floral visitors (*e.g.* florivores, nectar thieves) that consume substantial amounts of flower tissues, alter nectar availability and degrade pollinator attractiveness properties, which may ultimately reduce plant reproductive success (Galen 1999; McCall & Irwin 2006; Irwin *et al*. 2010; Moreira *et al*. 2019). Consequently, plants have developed a set of defense mechanisms and strategies to avoid or, at least, minimize the damage produced by floral enemies. Although some of these defensive systems have generally evolved to deter herbivores of vegetative tissues, they are also commonly and efficiently used to protect reproductive organs, even produced only during the flowering period in some species (Agrawal 1998; Karban & Baldwin 2007; Dutton *et al*. 2016). For example, indirect defense provides protection against herbivory and, in some cases, exclusively against florivory and nectar-thieving from flowers via mutualistic interaction with natural predators (Heil & McKey 2003; Trager *et al*. 2010; Lortzing *et al*. 2016).

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Myrmecophily is a pervasive form of indirect defense against herbivores of vegetative and reproductive organs. Ant-plant mutualistic interactions are biogeographically widespread and present across a diverse range of plant taxonomic groups (Davidson *et al*. 1989; Heil & McKey 2003; Rico-Gray & Oliveira 2007; Trager *et al*. 2010). The production of nectar to attract plant defenders (*i.e*. unrelated to pollinators; Heil 2011) is the most prevalent mechanism of ant-mediated indirect defense in myrmecophytic plants (see Weber *et al*. 2015). Secretory structures that excrete patrolling-related nectar and other chemical compounds as feeding resource for ants are often extrafloral (extrafloral nectaries), but they can also be located within flowers (extranuptial nectaries, *sensu* Delpino 1886; see also Mesquito-Neto *et al*. 2020 and references therein). Ant protective action, however, can concomitantly involve several types of conflicts for plants in terms of pollination (*i.e*. ant-pollinator conflict: Willmer & Stone 1997; Ness 2006; Assunção *et al*. 2014; Villamil *et al*. 2019). First, ants are mostly non-pollinating insects that may consume floral nectar and collect pollen without providing pollination services (Hölldobler & Wilson 1990; Rico-Gray & Oliveira 2007; Villamil *et al*. 2019). Second, ants may hinder pollination by repelling or directly attacking insect pollinators, thus ultimately reducing host plant's reproductive success (Willmer & Stone 1997; Byk & Del-Claro 2010; Assunção *et al*. 2014; Ibarra-Isassi & Oliveira 2018; Santos & Leal 2019). In brief, myrmecophilous plants face a two-fold trade-off as ant-mediated protection against floral enemies raises two non-exclusive ant-pollinator conflicts.

From an evolutionary perspective, it is expected a defense strategy to be potentially selected when producing a positive net cost-benefit balance for plant fitness (*i.e*. optimal defense theory: Stamp 2003); that is, by reducing the ant-pollinator conflict without compromising defense against floral enemies. In this regard, a wide set of mechanisms have been related to avoidance of ant visitation to flowers and pollinator deterrence during anthesis. For example, some species have physical barriers as pedicels or spines on corollas, calyces or floral pedicels that prevent ant movement (*e.g. Stephanotis*; reviewed in Willmer 2011). Other myrmecophilous plants produce different ant-repellent floral volatile compounds, mostly during the anthesis, to reduce ant access to flowers only when are open and functional (Willmer & Stone 1997; Raine *et al*. 2002; Junker *et al*. 2007; Ballantyne & Willmer 2012). Still, in some myrmecophilous species ant protection against floral enemies improves plant reproductive success without limiting ant access to open flowers. Pollination by large-sized pollinators (*e.g*. vertebrates) that are hardly influenced by patrolling ants could potentially account for this process. For example, *Passiflora coccinea* is a hummingbird-pollinated Neotropical myrmecophilous plant (Fig. 1) in which ant protection against floral nectar thieves does not interfere with pollinators and significantly increases seed production in ant-visited flowers when compared to ant-removed flowers (Leal *et al*. 2006). However, flowers of *P. coccinea* are also visited by pollen-collecting bees that do not contact stigmas (Storti 2002), but nothing is known about the ant protective role against these floral visitors and whether flower-visiting ants access anthers. Therefore, further research is required to unravel the ant intrafloral activity and its interference with the ant-pollinator conflict in systems wherein ants access flowers.

Here, we combine an observational approach with experimental manipulation of insect visits on reproductive (anthers and stigmas) and non-reproductive (bracts, corona and perianth –petals and sepals–) structures of *P. coccinea* flowers to determine whether non-pollinating protective ants access anthers and show a selective defense response among visitors on the different floral structures. Specifically, we expect ants (1) to never access reproductive structures despite some pollen-collecting bees visiting anthers without providing pollination service and, consequently, (2) to exclusively protect flowers against potential nectar-thieving visitors landing on non-reproductive structures. Evidence in this sense would potentially explain a reduced ant-pollinator conflict in flower-visiting ant-mediated protection against floral enemies.

Materials and methods

Species and study site

The study was conducted in August 2019 along a 2-km transect at the edges (0 – 20 m towards forest interior) of a 7,000-ha natural fragment of a tropical moist broadleaf forest of southern Amazonia adjacent to pasture areas for cattle located at Fazenda São Nicolau, Cotriguaçu, Mato Grosso, Brazil (9°51'21" S - 58°14'52'' W; Fig. S1 in supplementary material). The area is characterized by different land uses resulting of human perturbation, wherein pasture lands and reforestation areas are interspersed with secondary forest patches bordered by larger areas of primary forest (Rodrigues *et al*. 2011). The climate in this area is classified as Aw, hot and wet, according to the Köppen's system, with mean annual temperature of 24 ºC and precipitation of 2,300 mm (Rodrigues *et al*. 2011).

Passiflora coccinea Aubl. (Passifloraceae) is a Neotropical and evergreen woody vine inhabiting disturbed areas of humid forests and savannas from Central America to southern Amazon (Pio-Corrêa 1978; Ribeiro *et al*. 1999; Fischer & Leal 2006). Flowering spans between July and February, and blooming individuals open a few hermaphroditic, nectariferous and red disc-shaped individual flowers daily, about 7-12 cm in diameter, with three external bracts, five sepals, five petals in an alternate arrangement with sepals, five anthers and a superior ovary with three stigmas raised on an androgynophore surrounded by a corona with multiple filaments (Storti 2002; Fig. 1A-B). *P. coccinea* produces a few extrafloral nectaries on the leaf blade and a set of extranuptial nectaries located on the border of floral bracts, which are visited by more than 20 ant species, mostly of the genera *Camponotus*, *Crematogaster*, *Ectatomma* and *Pseudomyrmex* (Wirth & Leal 2001; Leal *et al*. 2006). Flowers open in the predawn and last fully open and functionally active during a few hours, closing before midday (Storti 2002; Fischer & Leal 2006). The hummingbird species *Phaethornis superciliosus* pollinates flowers when collecting nuptial nectar (Fig. 1C), located in chambers protected by the corona, contacting its head with the reproductive structures (Storti 2002; Fischer & Leal 2006). A few insects also visit flowers, mostly a few pollen-collecting bees and nectar-thieving butterflies that do not provide pollination service (Storti 2002; Fig. 1D-F). *P. coccinea* is a xenogamous and self-incompatible species, dependent on hummingbird-mediated cross-pollination for seed production (Storti 2002).

Data collection

Along six consecutive days in the morning (between 7:00 and 11:00) we observed floral visitors (*i.e.* pollinators, pollen-collecting bees and nectar thieves) and ant defense response on 52 flowers of 18 flowering individuals.

Figure 1. Flowers and individuals of *Passiflora coccinea* used in the study (**A, B**), with detailed images of some floral visitors (**C** – *Phaethornis superciliosus*; **D** – a nectar-thieving butterfly; **E, F** – pollen-collecting bees) and ant attack on bees used during the experiment on the different floral structures (**G** – corona; **H** – bracts; **I** – perianth; **H** – reproductive structures).

The ants present on each individual were collected and their identification matched the ants found in other studies conducted in the same area (*e.g.* Dáttilo *et al*. 2014; Vicente & Izzo 2017). However, we did not record which ant genera are more aggressive or account for most of attacks. Here, we explicitly focused on the ant defense response between non-reproductive and reproductive floral structures, regardless of specific defense response across different ant genera.

The observations of floral visitors were conducted by ourselves by means of either direct observations or digital video recording using 2 second time-lapse sequence photography (Nikon D7100, AF-P 70-300mm F/4.5-6.3G, Nikon Group, Japan). Direct observations were conducted during 10-min periods between 7:00 and 9:00 at a distance of ca. 1 m from the focal flower to give a total of five or six observation continuous periods per day and 34 periods, totaling 340 min (*ca*. 6 h). Observations with video camera followed the same protocol as direct observations, giving a total of three observation periods per day between 9:00 and 11:00 (18 periods, totaling 180 min; 3 h). During each observation period we noted the number and taxonomic group of floral visitors (species were not identified, but *Phaethornis superciliosus*) and floral structure visited (*i.e.* bract, corona, perianth –petals and sepals–, and reproductive structures –anthers and stigmas–). Since we focused on ant defense response, a visit was exclusively recorded when a floral visitor landed on any of floral structures. Thus, hummingbird's tongue- or bill-mediated contacts on the reproductive organs recorded were not ultimately considered in our analysis, as hummingbirds hover during their visits and are not influenced by patrolling ants (Leal *et al*. 2006). As flowers of *P. coccinea* are large and each of floral structures clearly conspicuous, it was easily possible to distinguish visits contacting each of these parts. For each visit, we also recorded whether ants attacked or not, considering attack as any physical interaction between ants and floral visitors. We considered physical interaction instead of merely recording defense-related ant behaviour changes or movements because of four reasons: (1) some visits were only sporadic, (2) ant movements on the flowers are dynamic and hardly related to aggressive behaviour, (3) all observed ant species were known as aggressive against herbivores in other systems, and (4) to avoid false positives.

However, this procedure could instead lead to false negatives, as some floral visitors may leave the flower before any physical interaction with ants, especially visits on reproductive structures. To avoid this limitation, we conducted an experiment in addition to the natural observation of floral visitors and ant response by simulating floral visits on the different floral structures. To do this, we used recently dead bee individuals (species was not identified) from a species visiting *P. coccinea* flowers previously collected and kept in small lab sterile bottles. The bees were carefully located on the different floral structures of six flowers from six different individuals (n = 24 observations) for at most five minutes to determine the ant attack and removed after this occurred (*i.e.* the first physical ant-bee interaction occurred; Fig. 1G-J). Any potential influence of using dead bees instead of living ones on ant attack was similar in the different floral structures. Besides recording the presence or not of ant attack, we quantified the time elapsed since we located the dead bee on a given floral structure and ant-bee physical interaction.

Data analysis

To test for significant differences in the ant attack (*i.e.* ant-visitor physical interaction) between floral visitors and structures in our observational approach, we fitted a Generalized Linear Mixed Model (GLMM). We included floral visitor and floral structure as fixed factors in the model. We also considered the variation within plants by adding this variable as a random factor in our model. Thus, we included a statistical control of this source of variation. A full model should also include the interaction floral visitor × floral structure, wherein a significant interaction indicates a differential effect of the floral structure on ant defense response depending on the floral visitor. However, the low number of samples (n = 55 floral visits) and the number of interactions (n = 12; 3 floral visitors × 4 floral structures; see Results) did not let us get a deviance, a reliable test value or a *p*-value for the interaction. Either way, both the model we ran and the full model showed ultimately the same results for floral visitors and floral structures. We considered a binomial (presence or absence of ant defense response) error distribution using the *MASS* package (Venables & Ripley 2013) in R software (R Development Core Team 2018). The model was analyzed using the restricted maximum likelihood (REML). We also performed post-hoc pairwise comparisons to determine significant differences in ant defense response among floral structures using the *lsmeans* package (Lenth 2016).

For the experimental design we did not conduct any analysis since the results were totally contrasting between reproductive organs and the other floral structures (0 vs 100 % of presence in ant defense response, respectively; see Results). Instead, we tested for significant differences in the time elapsed in the ant defense response between non-reproductive floral structures (bracts, corona and perianth) by fitting a Generalized Linear Model (GLM). We considered a Poisson error distribution and performed post-hoc pairwise comparisons to determine significant differences in time elapsed in the ant defense response among floral structures as explained above.

Results

We recorded four taxonomic groups of floral visitors (bees –Hymenoptera–, bugs –Hemiptera–, butterflies –Lepidoptera– and hummingbirds) comprising 64 visits to

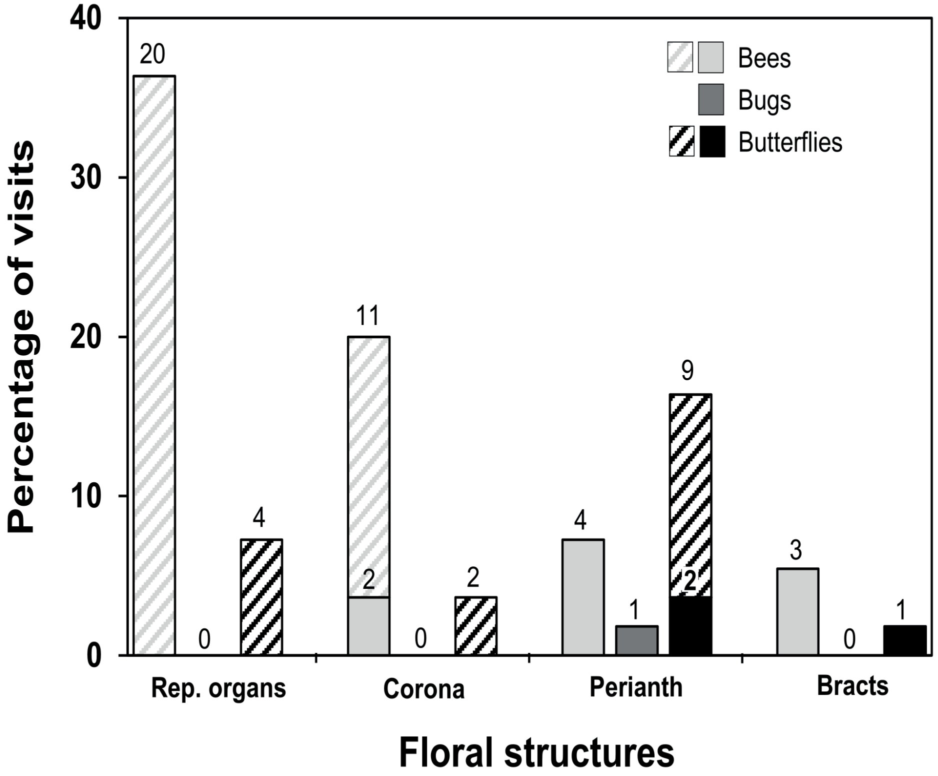

Figure 2. Percentage of visits carried out by the different floral visitors (excluding hummingbirds, see Materials and methods) on each of floral structures of *Passiflora coccinea* flowers. Solid bars show visits attacked by ants whereas hatched bars show non-attacked visits. Total number of visits are represented on each bar.

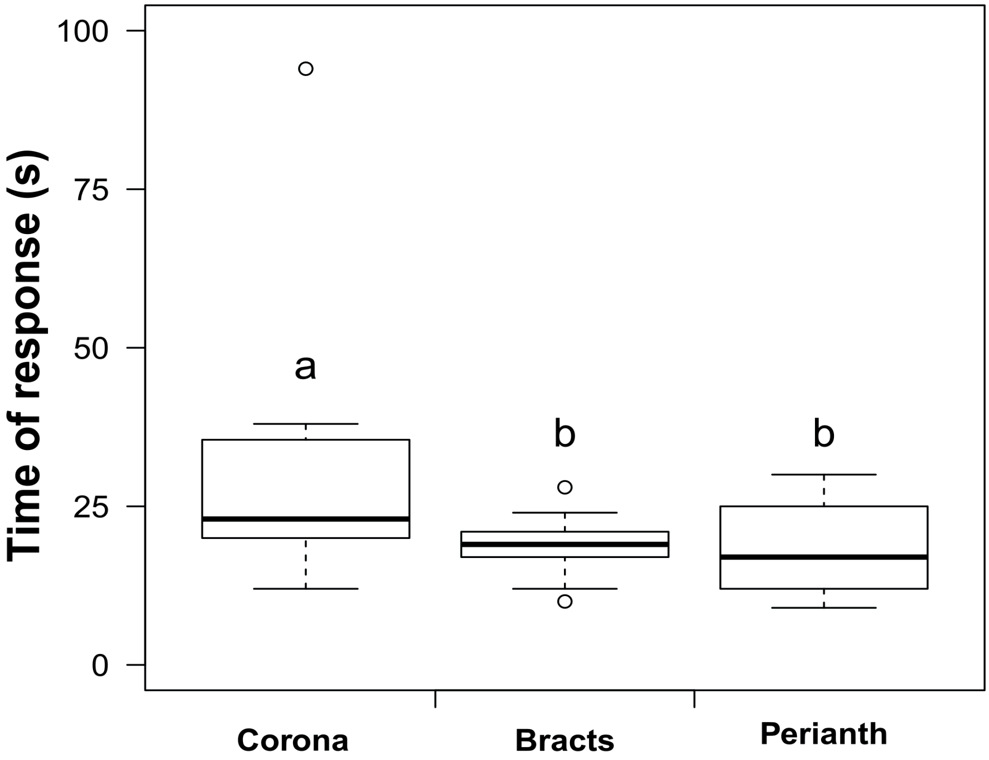

Figure 3. Box-plots showing medians and quartiles of time elapsed for the first ant attack, measured as the time the first physical contact between ant and bee occurred, across floral structures of *Passiflora coccinea* flowers. Different letters show significant differences ($P < 0.05$).

flowers of *P. coccinea*. However, we excluded hummingbird visits (nine visits) and exclusively focused on floral visitors landing on flowers as explained above (see Materials and methods). Thus, pollen-collecting bees (69 % of visits) and nectar-thieving butterflies (29 %) species were the most frequent visitors, whereas bugs only comprised one visit (Fig. 2). Reproductive organs were the most visited floral structure (44 % of visits), followed by the perianth (25 %), the corona (24 %), and bracts (7 %). Bees were the most recurrent visitors on reproductive organs, butterflies usually landed on petals and occasionally contacted reproductive organs, whereas the only visit reported by bugs was on sepals.

We recorded four ant species in flowers of *P. coccinea*: *Camponotus femoratus*, *Crematogaster levior*, *Azteca chartiflex* and *Pheidole gertrudae*. In our study observational, we noted thirteen attacks to floral visitors (*ca*. 20 % of visits). However, ant attack significantly differed between floral visitors ($\chi^2 = 12.36$, $P = 0.004$) and structures ($\chi^2 = 39.16$, $P < 0.001$). Most interestingly, floral visitors contacting the reproductive organs did not suffer any attack and only 10.5 % of the visits to the corona were defended (Fig. 2). Otherwise, all visits to bracts and 50 % to perianth entailed ant attack (Fig. 2). We found significant differences in ant defense response between bracts and perianth ($P < 0.001$) and between these structures and reproductive organs or corona ($P < 0.01$ for all pairwise comparisons). Our experiment reinforced the differences in ant defense response between reproductive and non-reproductive structures. For all experimental replicates, the reproductive organs were never defended as ants show a consistent behavior and did not ever access these structures, whereas bracts, corona and perianth always were. Mean time that ant attack took to appear, measured as the time the first physical interaction between ant and bee occurred, was significantly about two-fold longer in the corona than in bracts and perianth, which did not show any difference in time of attack (Fig. 3).

Discussion

By combining an observational survey with an experimental approach, our study shows compelling novel evidence that floral visitors that land on flower reproductive organs of *P. coccinea* are never attacked by ants, as they do not ever access these structures. Thus, differential ant defense response among floral structures with no access of ants to pollen is a relevant process across the diverse and widespread interaction between ants and plants. In this context, the ant's inability to attack floral visitors on anthers and stigmas may be regarded as an important adaptative mechanism in *P. coccinea* to ameliorate potential conflicts to pollination of flower-visiting non-pollinating ants without altering the defense against potential floral enemies visiting non-reproductive structures that do not provide pollination services.

Previous studies have shown that ants seldomly access flowers across extrafloral nectary-bearing plants (see Villamil *et al*. 2019 and references therein), whereas differential ant attack among floral structures has so far been overlooked. In *P. coccinea*, this activity may fairly be related to the specialized hummingbird-mediated pollination system. While foraging flowers, individuals of *Phaethornis superciliosus* hover to collect nectar and are not chased away by ant presence (Fischer & Leal 2006). Therefore, ant access to flowers does not involve indirect ecological costs in terms of pollinator deterrence, overcoming one of the main ant-pollinator conflicts. Ant activity within flowers entails a benefit by averting nectar-thieving butterflies to land on the corolla or, accordingly to our results, chasing them away once on the petals (Leal *et al*. 2006). In contrast, we also observed some pollen-collecting bees foraging on anthers that were not attacked by ants. However, an important caveat of our study is that we lack data regarding differential bee visitation rates between ant-visited and ant-removed flowers. In this regard, ant presence in flowers could still deter some bees and reduce their visitation rates and/or duration (LeVan & Holway 2015; Ibarra-Isassi & Oliveira 2018). Likewise, it would be interesting to determine whether this pollen theft can ultimately compromise the male fitness by reducing siring success. Although pollen is usually overproduced across animal-pollinated plants and male function is limited by the number of mating events (Burd & Callahan 2000; Teixido *et al*. 2016), native pollen thieves can still reduce male pollination components (Hargreaves *et al*. 2010). Other stimulating topic would be to disentangle the ant defense response against hemipteran species, which may be more abundant in our study system. Although most hemipterans are herbivores (*e.g.* stink bugs, Pentatomidae), some species prey upon pollinators (*e.g.* assassin bugs, Reduviidae), disrupting plant-pollinator mutualisms (Benoit & Kalisz 2020). Hence, ant attack against these predators could entail an additional benefit to plants beyond defense against herbivores. Either way, our results suggest that ant interaction in *P. coccinea* maximizes protection against nectar thieves without incurring the potential negative impacts of non-pollinating ant access to pollen.

The most plausible explanation for the eventual process that regulates the selective ant defense response among floral structures may be related to the corona of filaments of flowers. Flower nuptial nectaries in *Passiflora* species are in chambers protected by this floral structure (Durkee *et al*. 1981; see also Storti 2002 for *P. coccinea*). This protection could limit the access to nectar-thieving insects although, as described, some butterflies still consume nectar of *P. coccinea* flowers with their mouthparts by landing on perianth (see also Leal *et al*. 2006). Otherwise, the corona in flowers of *P. coccinea* seems rather to be a structure that acts as a physical barrier to simultaneously protect the accession of ants to nectar and anthers. Physical barriers such as floral pedicels and spiny or hairy corolla surfaces have been broadly related to reduction in ant visitation to flowers during anthesis by hampering their walking (Harley 1991; Willmer 2011). We suggest that the corona restricts ant access to nectar and pollen and, subsequently, pollination interference, but also ant protection against pollen-collecting bees and other illegitimate visitors on reproductive structures of *P. coccinea* flowers, but corona-removing experiments are required to take solid conclusions.

Together with the corona role, the emission of floral volatile compounds could disentangle the differential ant protection among floral structures to some extent. Mounting evidence reveals that ant-deterrent chemical signals in floral tissues are relatively common in myrmecophytic plants (*e.g.* Guerrant & Fiedler 1981; Willmer & Stone 1997; Ness 2006; Agarwal & Rastogi 2008; Ballantyne & Willmer 2012). In *Vachellia* (formerly *Acacia*) flowers, the repellent effect can even occur as a response to floral volatiles from pollen, averting ants just temporarily while flowers remain open and functional (Willmer *et al*. 2009). It is broadly known that some passion flowers produce nectar with volatile compounds related to pollinator attractiveness such as butterflies, bats and hummingbirds (Durkee *et al*. 1981; Varassin *et al*. 2001; DellaCuna *et al*. 2018; see also Fischer & Leal 2006 for *P. coccinea*). Interestingly, Konstantinidis *et al*. (2010) found that flowers of *Passiflora incarnata* produce chemicals in tissues, but not in floral nectar, that can effectively repel ants. Following these assumptions, a similar process to pollen chemical-mediated ant deterrence reported among flowers at contrasting phenophases of *Acacia* species (Willmer *et al*. 2009) could take place among reproductive and non-reproductive floral structures within flowers of *P. coccinea*.

Lastly, the lack of ant aggressive behaviour on insects contacting anthers and stigmas may instead be related to larger spatial separation between reproductive parts and extranuptial nectaries, which could potentially reduce the chances of nectar-thieving from the latter structure. In this regard, an inherent indirect cost to the production of nectaries associated to ant-guarding is an increase in the frequency of nectar thieves foraging these nectaries (Aguirre-Jaimes *et al*. 2018). However, an exclusive ant patrolling of extrafloral or extranuptial nectaries in plants with floral nectar appears to serve more as a diversion of non-pollinating ants from visiting flowers rather than a protective role against floral enemies (*i.e.* distracting hypothesis: see Villamil *et al*. 2019 and references therein). In agreement to our results, the secretion of extranuptial nectaries and the ensuing interaction with ants in *P. coccinea* has been differently suggested as a defensive function for flowers against nectar thieves, which significantly increases seed production in individual plants (Leal *et al*. 2006). Therefore, the presence of ants in *P. coccinea* not only protects flowers against potential floral enemies but also does not interfere with potential pollinators.

Ants of the genus *Pheidole* and, especially, *Camponotus* and *Crematogaster* have been previously reported to be frequent and abundant in *P. coccinea* (Wirth & Leal 2001; Leal *et al*. 2006). All these genera are associated to extrafloral/extranuptial nectaries and show adaptations to a liquid diet and to occupancy and foraging on the vegetation strata (Dáttilo *et al*. 2014). The four ant species identified in our study also show an extremely natural aggressive behavior by defending the area close to the food source (Dáttilo *et al*. 2014; Vicente & Izzo 2017). Overall, these assumptions suggest that the ant species reported in our study are defenders equally efficient against floral enemies of *P. coccinea*.

In conclusion, our results reinforce the assumption that the production of extranuptial nectaries in *P. coccinea* is associated to ant-mediated indirect defense to protect flowers against potential nectar-thieving caused by illegitimate floral visitors. More interestingly, our study demonstrates that the visits of insects on reproductive organs are not defended by extranuptial nectary-associated patrolling ants, as they did not ever access these structures. As a consequence, ants do not access pollen, thus avoiding potential interferences with pollination. This inability in ant defense response may consequently be an adaptative process to prevent an ant-pollinator conflict in this species. The eventual mechanism that regulates this process seems to be closely linked to the corona of filaments that protects nectar chambers in *P. coccinea* flowers and congeneric species. The role of the production of chemicals in floral tissues (*e.g.* ant-repellent pollen) raises interesting open questions that deserve further attention.

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