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Anti-bacterial function in the sexually dimorphic pollinator rewards of *Clusia grandiflora* (Clusiaceae)

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Abstract Many species of the dioecious, neo-tropical plant genus *Clusia* secrete a viscous, hydrophobic resin from glandular tissues in both male and female flowers. This substance is readily gathered by meliponine and euglossine bees for whom it most often serves as the sole pollinator reward. Bees use *Clusia* resin as a nest-building material. As such, resin clearly serves an indispensable mechanical function. However, resins with antimicrobial properties may also serve to reduce the risk of pathogenesis in the nest. If resin-gathering apids benefit from antimicrobial properties in nesting materials and are able to discern these characteristics in the forage they gather, one might predict that the resin reward presented in *Clusia* could have evolved under selection for both mechanical and antimicrobial properties. In dioecious species, where females and males each present a resin reward, selection regimes may differ between the sexes with the result that resin form and function diverge. We investigated both the form and function of the male and female pollinator reward resins of *Clusia grandiflora*. Using thin-layer chromatography (TLC), we compared the chemical compositions of floral resins from five widely separated populations of this species growing in southeastern Venezuela. We found that male and female resins exhibited a marked chemical dimorphism, with females having two major TLC-resolvable fractions and males having seven. This dimorphism was stable: there were no component differences between populations in either sex. Using a disk-diffusion technique, we surveyed the same resins for antimicrobial activity using assay microorganisms isolated from eusocial meliponine bees. Both male and female *Clusia grandiflora* resins had pronounced but relatively directed antimicrobial activity: both were toxic to 10 of 11 Gram-positive bacteria, 7 of 15 Gram-negative or variably-staining bacteria, 0 of 3 yeasts, and 0 of 3 filamentous fungi. Again with the disk-diffusion technique, we performed more detailed tests of resin bioactivity using two Gram-positive honeybee associates, *Paenibacillus larvae* and *P. alvei*, as model pathogens. Both male and female *C. grandiflora* resins were highly toxic to these honeybee pathogens. Female resin, however, produced zones of inhibition with more than twice the mean diameter of those produced by the male resin. This divergence in form and function of the *C. grandiflora* pollinator reward resins could be in response to different selective regimes as mediated by the pollinating insects.

Key words *Clusia* · *Paenibacillus* · Dioecy · Resin · Pollinator reward

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Introduction

Most entomophilous flowering plants present their pollinators with some form of reward (Faegri and van der Pijl 1971). This is most often an energy- or nutrient-rich substance such as pollen, nectar, or nutritive oil (Dafni 1992). A small number of angiosperms, however, offer pollinator rewards that are non-nutritive resins (Armbruster 1984). This rare phenomenon is limited to a few bee-pollinated tropical genera. One of these is the neotropical genus *Clusia*, many of whose approximately 250 species secrete a viscous, hydrophobic resin from glandular tissues in the flowers. This substance is highly attractive to a variety of meliponine and euglossine bee species for whom it serves in most cases as the sole pollinator reward (Bittrich and Amaral 1996, 1997).

Bees use *Clusia* resin as a nest construction material and for many species this substance serves an indispensable mechanical function (Roubik 1989). Chemical analyses of several apid-bee nests in Venezuela indicate that the most abundant components are of clusiaceous origin (Tomás-Barberán et al. 1993). Resins are used to bind together solid materials such as sand, hair, and

twigs to form the nest walls. They are also mixed with endogenously produced waxes or used in pure form to shape the nest entrance, larval chambers, and storage pots. For nest-building apid species, resinous substances are critical structural materials. In addition to their mechanical utility, however, bees may derive another less apparent benefit from resins when these materials are toxic to potential nest pathogens. The bee nest is both a repository for energy and nutrient-rich forage and a protected site for the production of new bees (Roubik 1989). As such, the potential for pathogenic attack is presumably significant. Although little is known about the diseases of wild bees, the nests of the honeybee, *Apis mellifera*, are vulnerable to a number of microbial pathogens including viruses, bacteria and fungi (Bailey and Ball 1991). Nest construction materials that deter microbial growth may provide some measure of protection against pathogenic attack.

Insect pollinators are known to be a selective force on numerous floral characteristics. These include such diverse features as petal color, corolla shape, pollen presentation, and pollinator reward (e.g., Stanton et al. 1989; Campbell et al. 1991; Thomson and Thomson 1992; Mitchell 1993). In the case of the resin rewards presented by *Clusia* species, apid-bee pollinators may have provided a selective force that has shaped resin chemistries associated with particular resin functions, both mechanical and antimicrobial. If apids realize some benefit from using antimicrobial materials in their nests and they seek resins having this property, antimicrobial activity may have been selected for in *Clusia* resins along with such mechanical characteristics as malleability and hydrophobicity.

The majority of *Clusia* species are dioecious, males and females being separate individuals. This condition frees plants from many of the constraints of a hermaphroditic breeding system and can lead to a more independent evolution of male and female functions (Nicotra 1998; Meagher and Antonovics 1982). Both ecological specialization of the sexes and the divergence of secondary sexual characteristics are possible outcomes. In plant taxa where pollen flow is dependent upon an insect vector, characters associated with pollen export, that is, male function, and characters associated with pollen receipt, a female function, may respond differently to insect-mediated selection environments. An example of this occurs in the partly dioecious orchid genera *Catasetum* and *Cycnoches* where males and females of several dioecious species have strongly contrasting floral forms (Dodson 1962; Dressler 1968). This is apparently the result of selection by pollinating bees who seek to avoid re-visiting flowers with an appearance similar to the male orchid where a pollinium has been forcibly attached (Romero and Nelson 1986).

In the genus *Clusia*, as in *Catasetum* and *Cycnoches*, pollen transfer is effected by relatively specialized apid-bees. As a secondary sexual characteristic associated with flowers of both sexes, the resin pollinator reward in male and female *Clusia* plants may well have evolved in differing insect-mediated selection environments. This paper reports on a study of the antimicrobial properties of the *Clusia* pollinator reward system in light of the consequences that differing selective regimes may have had for the evolution of this secondary sexual feature. In particular we focus on the resins of *Clusia grandiflora*, a species native to the Amazon and Orinoco drainages of South America. Both male and female flowers of *C. grandiflora* produce abundant quantities of resin, 500–700 mg per flower, as the sole pollinator reward. With regard to this reward system, we address two general questions. First, are *C. grandiflora* resins microbicidal and if so, what is the range of their bioactivity? Second, do male and female resins exhibit structural and/or functional differences that could be the result of their evolution under differing selective environments?

Though there have been several studies of *Clusia* secondary metabolite chemistry (de Oliveira et al. 1996; Cerrini et al. 1993; Gustafson et al. 1993; Delle Monache et al. 1991a, 1991b), none have addressed the microbiological aspects of resin use by bees. This study is the first to directly test an important apid-bee pollinator reward for a possible role in disease control. Our approach to the above questions began by first assessing the chemical makeup of male and female *C. grandiflora* resins. We collected resin samples from five populations of this species occurring in southeastern Venezuela. Then, using thin-layer chromatography (TLC), we separated resins into their major component fractions. We made comparisons between males and females within each population as well as same-sex comparisons between populations. Next, we performed bioassays of resin antimicrobial activity for both sexes. These were carried out in three stages. We first surveyed resin bioactivity against a collection of microorganisms that had been isolated from eusocial meliponine bees. Then we performed a more detailed set of assays using specific microorganisms including two well characterized bee pathogens. Finally, we tested specific resin fractions for antimicrobial activity.

Materials and methods

Collection site and study species

We collected *C. grandiflora* floral resins in southeastern Venezuela, within the boundaries of Parque Nacional Canaima, in an area known as the Gran Sabana (4°30′–6°15′N, 61°30′–62°W). *C. grandiflora* grows here as a terrestrially rooted tree in rocky riparian habitats. It has a wide but patchy distribution and populations are often separated by many kilometers of open grassland. Timing of *C. grandiflora* florescence is variable and appears to be strongly influenced by recent precipitation patterns. Both male and female flowers are radially symmetrical, 15–18 cm in diameter, with large white-to-pinkish petals. The resin-secreting tissues of both sexes are sterile stamens or staminodia. In the male, staminodia lie at the center of the flower and are surrounded by a ring of fertile stamens. In the female, staminodia encircle the prominent pistil. Resin is secreted liberally in the hours following anthesis which are midnight to 1 a.m. for the male and 5–6 a.m. for the female

(authors, personal observations). Resin accumulates on the surface of the staminodia and forms a continuous 500- to 700-mg mass of transparent yellow liquid. Bees gather this material by rolling it into balls which are passed to the corbiculae for transport. Nectar is not secreted by either sex. Flowers of both sexes are short-lived, typically losing their color the day following anthesis. Voucher specimens of both *C. grandiflora* sexes from the Gran Sabana are available through the Herbario Nacional de Venezuela (VEN), (accession numbers VEN 294991, VEN 294992), and through the Herbario Regional de EDELCA, San Ignacio Yuruaní, Bolívar, Venezuela.

Resin collection and chemistry

We collected resins from five populations of *C. grandiflora* along a 150-km transect in Parque Nacional Canaima in October and November 1997. Resins were removed directly from flowers with a spatula and transferred to Teflon-stoppered glass vials. These samples were returned to the University of Alaska Fairbanks, for analysis where we assessed both between-sex and between-population differences in resin chemistry. We used TLC to separate resin components for comparisons. Thin-layer chromatograms were run in parallel on a single, aluminum-backed silica gel sheet (Silica gel 60 F_{254}, E. Merck, Darmstadt, Germany) using dichloromethane/acetone (92:8 for between-sex and 98:2 for between-population comparisons) for the solvent system. We prepared crude resin solutions of 1 mg ml^{-1} in dichloromethane and using capillary pipettes (Drummond Scientific, Broomall, Pa., USA), spotted 5 µl of each solution at the origins thus insuring that chromatographic comparisons were between equal resin masses. Separations were visualized using UV light at 254 nm and by treatment with 6% sulfuric acid/ethanol spray followed by heating.

Microbiological assays

Assays for antimicrobial activity in *C. grandiflora* floral resins occurred in three phases. The first two, an environmental isolate survey and an *Apis mellifera* pathogen assay, tested activity of whole crude resin. A third specific resin-fraction assay tested individual resin component groups for bioactivity. For all assays, the microbiological media we used were supplied by DIFCO (Detroit, Mich., USA).

Environmental microorganism isolation

In the first set of assays, we surveyed a collection of environmental microorganisms that were isolated as follows. We located the nest of a native eusocial meliponine, *Paratrigona anduzei*, in Mucuy Bajo, Mérida, Venezuela in January 1995. We trapped single worker bees returning to the nest from foraging flights directly into Petri plates that contained either nutrient agar (NA), a broadly-selective heterotrophic medium, or potato dextrose agar (PDA), a common fungal medium. Each bee was allowed to walk across the surface for several minutes before being released. We performed eight such entrapments with each medium as well as three air-exposure controls in which we imitated the movements of bee entrapment. We incubated all plates at ambient temperatures (23–28°C) for 4 days at which time bee-exposed plates and controls were compared. Growth on control plates, both NA and PDA, was mostly filamentous fungi and generally sparse. Individual colonies from the bee-exposed plates that, based on colony morphology, were distinct from growth on the controls were transferred to the appropriate medium and returned to the University of Alaska Fairbanks. Through further transfers and separations of mixed cultures, we were able to isolate 32 microorganisms as pure cultures. Based on microscopic and Gram stain characteristics, we placed them in the following categories: 11 Gram-positive bacteria, 15 Gram-negative/variable bacteria, 3 yeasts, and 3 hyphal fungi. Each culture was subsequently cryo-suspended in a mixture of growth medium, glycerin, and water (2:1:1).

Apis mellifera pathogens

For the second set of assays, we obtained bacteria from the American Type Culture Collection (ATCC). As little is known of wild bee pathogens, we chose two well characterized honeybee nest associates as models. These were *Paenibacillus larvae* (ATCC #6344), the causative agent of American foulbrood and *P. alvei* (ATCC #9545) an opportunistic pathogen/saprophyte associated with European foulbrood (Shimanuki 1990). For comparisons, we also used two bacteria that are common mammalian commensals/opportunistic pathogens, *Enterococcus faecalis* (ATCC #19433) and *Staphylococcus aureus* (ATCC #25923). *E. faecalis* has also been identified as an environmental contaminant and opportunistic pathogen of honeybees (Bailey and Ball 1991).

Assay protocol

We conducted bioassays of whole crude *C. grandiflora* resins using these two sets of microorganisms. Our assay method was disk-diffusion: filter paper disks impregnated with a known mass of a test substance were placed on the surface of a solid medium that had been inoculated with a pure culture of assay microorganism. Growth occurred evenly except where inhibitory substances had diffused across the surface of the medium. The diameter of the inhibition zone was measured and used as a measure of antimicrobial potential.

Except in the case of the filamentous fungi, each assay was conducted using the same protocol as follows. We prepared acetone solutions of each crude resin in a septum-stoppered glass vial. These were of known concentration, typically 1–10 mg ml^{-1} depending on what final resin-mass/disk was required. Using a 100 µl syringe (Hamilton Co., Reno, Nev., USA), we applied appropriate volumes of resin solutions to 3.5-mm-diameter filter paper disks (Whatman #5, Whatman Labsales, Hillsboro, Ore., USA) to achieve the desired resin-mass/disk. The solvent was removed by drying in an air stream. For comparison, we included disks prepared with two stock antibiotics, ampicillin and chloramphenicol (Sigma Chemical Co., St. Louis, Mo., USA), in the *Paenibacillus larvae* and *P. alvei* bioassays. These were mixed as water and ethanol solutions respectively and were applied by syringe to achieve a final mass per disk equal to the resin test masses.

We prepared the inoculum for each assay by allowing a microorganism to grow in liquid medium for 12–24 h at different controlled temperatures, depending on the growth requirements of the organism. For *Paenibacillus larvae*, the medium was brain-heart infusion broth and the incubation temperature was 37°C. For all other bacteria and the yeasts, the media were nutrient broth and potato dextrose broth respectively and the growth temperature was 25–30°C.

We made the equivalent solid medium for each assay by adding 1.5% agar and sterilizing: 20 ml of the sterile solution was poured into 100 × 15 mm glass Petri dishes and cooled, and then 200 µl of fresh liquid inoculum was pipetted onto the solid media and spread evenly across the surface until all trace of liquid had been absorbed into the agar. We then placed the resin-impregnated disks, the solvent controls, and the antibiotic disks when used, on the medium surface. We sealed and incubated the Petri dish at the appropriate temperature, 37°C for *P. larvae*, and 30°C for all other organisms. Incubation times were 24–48 h depending on the growth rate of the assay organism. Once confluent growth was evident, we measured the diameters of inhibition zones (if they occurred) to the nearest millimeter using calipers. Statistical analyses of results from the *A. mellifera* pathogen assays were performed using SYSTAT for Windows, Version 5 (SYSTAT, Inc., Evanston, Ill., USA). Two-tailed *t*-tests were used for two-way comparisons; MANOVA followed by Bonferroni pair-wise comparisons were used for multiple comparisons.

For the filamentous fungi assays, we prepared inocula by allowing a fungus to cover a PDA plate. We then cut a small block of agar out of this culture and placed it at the center of a fresh PDA plate. Resin-impregnated disks and solvent controls were placed in a circular pattern *c*. 2 cm from the fungal inoculum. During incubation, radial growth of the fungus then proceeded toward the disks and inhibition zones (if they formed) were measured.

Specific resin fraction assay

Finally, we performed an assay for activity of specific resin fractions using *Bacillus megaterium* (Carolina Biological Supply, Burlington, N.C., USA), an organism known to us to be susceptible to *C. grandiflora* resins. Using silica gel TLC strips approximately 100 mm in length, we spotted the origins with 25 μg of crude male or female resin in two side-by-side channels and ran them in 100% dichloromethane. The strip was cut in half lengthwise and one channel was developed with 6% sulfuric acid in ethanol. This served as the reference. The other channel was placed in a glass Petri plate and embedded in melted agar medium containing 2% soluble starch which had been inoculated with 5% of fresh liquid culture of *B. megaterium*, a starch-metabolizing organism. The culture was incubated at 30°C. until heavy bacterial growth was observed, then an iodine solution was poured onto the surface of the medium. A dark blue starch-iodine complex formed where starch persisted, that is, in the zones of bacterial inhibition. By comparison with the reference TLC strip, specific resin fractions, as separated by TLC, could be identified as being antibacterial.

Results

Resin chemistry

Using TLC, we compared male and female resin chemistries from five populations of *C. grandiflora* in the Gran Sabana of Venezuela. This analysis showed that there is a pronounced sexual dimorphism in resin chemical composition: male resins have seven major TLC-resolvable fractions; female resins have two (Fig. 1). At the same time, TLC indicated that there is no between-population variability in resin composition for either sex (Fig. 2). These observations were based on both UV and acid-development visualizations. Acid development, furthermore, provided a qualitative colorimetric comparison of resin components. We observed complete color uniformity between male resin components and between female resin components. This provided further evidence that there are no gross differences in resin chemistry between widely separated populations of *C. grandiflora*. Based on color intensities, the TLC showed slight variation in relative component abundance in one individual, from the Sierra de Lema population.

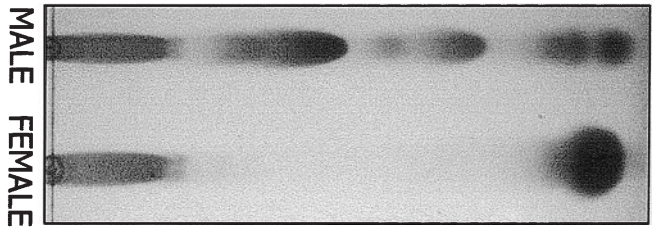

Fig. 1 Silica gel thin-layer chromatogram of whole male and female *Clusia grandiflora* pollinator reward resins run in dichloromethane/acetone 92:8 and photographed under UV (254 nm) light. All but the most polar fraction of both resins were bactericidal to *Bacillus megaterium* in specific-fraction assays

Environmental isolate survey

The initial phase of antimicrobial activity testing was a survey of 32 environmental microorganisms isolated from wild bees (Table 1). Positive activity for this set of assays was defined as a zone absent of all growth that was greater than two disk diameters, 7 mm. Resins of both sexes of *C. grandiflora* have antimicrobial properties. These appear, within the limits of resolution of the survey, to be most pronounced against Gram-positive bacteria. In addition, male and female resins show complete overlap in bioactivity: both had antimicrobial effects toward the same set of microorganisms.

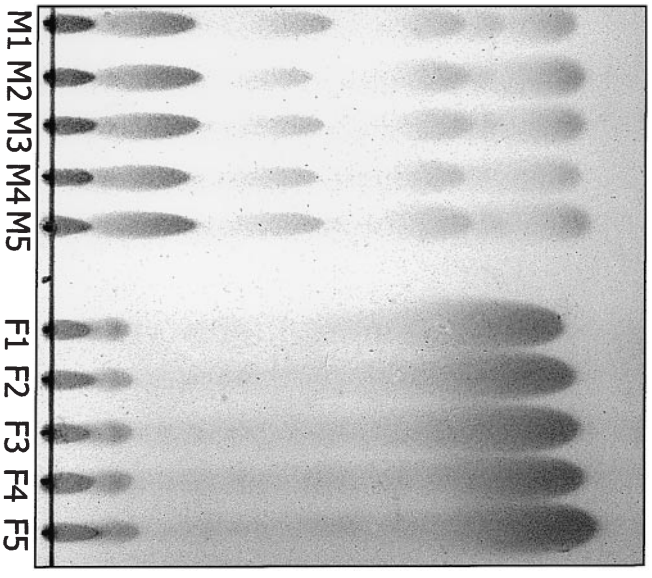

Fig. 2 Silica gel thin-layer chromatogram of whole male and female *C. grandiflora* pollinator reward resins from five populations in southeastern Venezuela run in dichloromethane/acetone 98:2, developed with 6% sulfuric acid/ethanol, and photographed under visible light (*M* male, *F* female, *1* Sierra de Lema, *2* Parupa, *3* Kuraverán Paru, *4* Quebrada Pacheco, *5* Yuruaní)

Table 1 Results from disk-diffusion assays of *Clusia grandiflora* resin bioactivity (200 μg resin per disk). Values are the number of assays in which activity was observed over the total number of assays

Assay microorganism class	Male resin	Female resin
Gram-positive bacteria	10/11	10/11
Gram-negative/variable bacteria	7/15	7/15
Yeasts	0/3	0/3
Filamentous fungi	0/3	0/3

A. mellifera pathogen assays

Through the results of the first phase of assays, it became apparent that *C. grandiflora* resins are generally toxic to Gram-positive bacteria. This motivated the second phase of assays in which we conducted more detailed tests using four Gram-positive bacteria (Table 2). Two of these, *Staphylococcus aureus* and *Enterococcus faecalis*, are common human commensals or opportunistic pathogens. The other two are honeybee-associated bacteria: *Paenibacillus larvae*, the organism causing American foulbrood and *P. alvei*, an organism known to attack weakened *A. mellifera* hives. Both resins showed pronounced toxicity to the assay organisms, with female resins generally exhibiting far greater antimicrobial activities than male resins. This is particularly true when they are assayed against the *Paenibacillus* bacteria.

Specific resin fraction assay

The results of the specific fraction assays showed which components of male and female resins are inhibitory to *B. megaterium*. Bioactivity in both male and female resin is concentrated in the less polar (more hydrophobic and mobile) fractions (Fig. 1). All but the single most polar fractions in both male and female resins are active. Bioactivity in the female is accounted for by a single hydrophobic fraction, and in the male, activity occurs in each of the six most non-polar fractions. The most polar (least hydrophobic and mobile) components of both resins show scant activity.

Discussion

TLC analyses of male and female *C. grandiflora* pollinator reward resins exposed a marked sexual dimorphism in resin chemistries: male resin had seven major fractions and female resin two (Fig. 1). This dimorphism was both stable and widespread in the Gran Sabana of Venezuela. For the five populations we sampled, there were no detectable between-population differences in chemical makeup of either male or female resins (Fig. 2). This implies that pollen flows freely between populations, that selection on resin chemistry is uniform across populations, or that some combination of the two processes is at work.

Our survey for bioactivity against 32 environmental microbes isolated from eusocial meliponine workers in Venezuela showed that both male and female *C. grandiflora* resins had pronounced antimicrobial activity. This activity was particularly evident against Gram-positive bacteria (Table 1). We saw no evidence of activity against fungi and markedly less against Gram-negative or variably staining bacteria. The results of this environmental isolate survey point toward a specific role for *C. grandiflora* resins as Gram-positive bactericidal agents. The most serious of the bacterial pathogens of honeybees, *Paenibacillus larvae* and *Melissococcus pluton*, are both Gram-positive organisms. Many of the secondary pathogens that attack diseased bee larvae are also Gram-positive: *Achromobacter eurydice*, *Bacillus laterosporus*, *P. alvei*, and *Enterococcus faecalis* (Bailey and Ball 1991).

Of the four Gram-positive bacteria we chose for our second series of more detailed assays, two were strict bee-associates, *P. larvae* and *P. alvei*. The other two we used are not normally bee-associated organisms. *S. aureus* is a mammalian associate and opportunistic pathogen; *E. faecalis* is a common mammalian gut organism but has also been isolated from bees infected with *M. pluton*. This set of assays produced three notable results. First, it shows that the chemical dimorphism in *C. grandiflora* resins underlies a pronounced difference in antibacterial potentials. This is particularly apparent in assays against the *Paenibacillus* bee associates where female resin inhibition zones were twice the diameter and five times the area of those produced by male resin (Table 2). As indicated by the specific resin-fraction assay, this is apparently not due to solubility differences, since both male and female resin activity is most pronounced in the less polar (more hydrophobic) fractions. Second, the antibacterial activities of *C. grandiflora* resins, and especially that of the female, are potent effects. When assayed against the *Paenibacillus* bacteria, crude female resin produced inhibition zones comparable to those of pure chloramphenicol at equal test masses. In these assays, both ampicillin and

Table 2 Results from 3.5 mm disk-diffusion assays of male and female *C. grandiflora* floral resins (*n.d.* no data)

Assay bacterium	Test mass (μg per disk)	Inhibition zone diameter (mm) ($-x \pm$ SE)			
		Male resin	Female resin	Ampicillin	Chloramphenicol
Staphylococcus aureus[a]	100	8.5 ± 0.1^{c}	14.2 ± 0.1^{d}	n.d.	n.d.
Enterococcus faecalis[a]	25	5.4 ± 0.1^{c}	7.7 ± 0.2^{d}	n.d.	n.d.
Paenibacillus larvae[b]	10	8.2 ± 0.2^{c}	18.9 ± 0.2^{d}	63 ± 5^{e}	22 ± 1^{d}
P. alvei[b]	5	8.1 ± 0.1^{c}	16.6 ± 0.1^{d}	0	24.8 ± 0.6^{e}

[a]Superscript letters in body of table indicate differences in two-tailed *t*-tests at $P < 0.0001$ ($n = 21$ female, 16 male)
[b]Superscript letters in body of table indicate differences in Bonferroni pair-wise comparisons at $P < 0.0001$ ($n = 21$ female, 18 male, 3 ampicillin, 3 chloramphenicol)

chloramphenicol appear to be more effective bactericides, but their comparison with *C. grandiflora* resin activity must be interpreted with solubility differences in mind. Chloramphenicol has measurable solubility in water whereas resin solubility is below the detection limits of normal gravimetric means. Disk-diffusion assays are run in an essentially aqueous environment: the compound being tested must diffuse through the watery interstices of the agar matrix. These assays then may substantially underestimate true physiological resin activity compared to the antibiotics. And third, *C. grandiflora* resin is decidedly toxic to two Gram-positive bacteria that are obligate honeybee associates. If, as we have assumed, these microorganisms are realistic models for the diseases of other apid bees, it appears that *C. grandiflora* resins, depending on how and where they are used in the nest, may be effective in controlling nest pathogens.

This study is the first report of potent antimicrobial activity in a non-trophic pollinator reward. These results suggest the intriguing possibility that the *C. grandiflora* pollinator reward system serves a two-fold purpose: besides the apparent mechanical utility of the resin, it also contains potent antibacterial substances. While providing apid pollinators with an indispensable building material, *C. grandiflora* may also be rewarding bees with chemical means to protect their nests from pathogenic attack. Although both sexes present bactericidal resin rewards, there is a marked dimorphism in the resins' compositions and in their activities. Given the high degree of uniformity in resin chemistries over a wide area, it would appear that these traits are adaptive and that male and female rewards have evolved under different selection environments. There are several plausible evolutionary scenarios that could have led to the current reward system. We consider two of them here.

Ancestors to *C. grandiflora* likely offered an antibacterial but monomorphic resin. This is the case today in other *Clusia* species of the Gran Sabana, such as *C. schomburgkiana* and *C. columnaris* (authors, unpublished work). In one scenario, male *C. grandiflora* resin chemistry remains conserved and the female diverges. In the Gran Sabana, present day populations of this species have approximately equal proportions of male and female trees. Male trees, however, produce 10–20 times the number of flowers that females do (authors, unpublished work). A representative riparian plant community in the Gran Sabana may contain a population of 100 adult *C. grandiflora* trees with an average male producing 50 flowers and an average female 10 flowers over the course of the flowering season. Since the flowers of both sexes contain 500–700 mg of resin, there could in one season be up to 2 kg of resin presented to pollinators from just one species of *Clusia* in the population. And most populations of *C. grandiflora* co-occur with two or three other resin-producing congeners. Pollinator visitation rates would then decline, possibly to the point where females are pollen limited. Because of their numerical inferiority, females will experience more intense selection pressure for particular resin characteristics than will males. In such a scenario, selection will favor those females which present a qualitatively different and more attractive pollinator reward. If the strength of antibacterial activity is a characteristic that bees assess when making resin foraging choices, individuals that present the more potent bactericide will be preferentially visited, thus driving a divergence in resin chemistry. The greatly enhanced bioactivity seen today in female resin may be the result of such selection. Potent antibacterial activity could provide the increment of attractiveness that effectively compensates for occasional over-abundances of male flowers. A similar scenario has been reported in Costa Rican forests where females of some dioecious tree species have been shown to produce up to 400% more nectar per flower then their male conspecifics (Bawa and Opler 1975). Here too, the authors propose that the greater attractiveness of the female reward compensates for the large numerical difference in flower output between the sexes.

In a second scenario, female resin chemistry remains conserved and male chemistry diverges. This could be the result of selection for a less viscous resin, one that is workable in the earliest morning hours. Male *C. grandiflora* flowers open shortly after midnight and secrete their entire resin complement in the hours before dawn. Pre-dawn temperatures in the Gran Sabana are cool enough that resins are quite stiff compared to their consistencies later in the day. If resin is a prized commodity among bees, selection pressures may have led to earlier and earlier foraging times as a means of gaining access to a limiting resource. Earlier foraging times select for earlier flowering times and of necessity more malleable resin. The expression of additional compounds may have become fixed in male resin because they decreased its viscosity. Indeed today we observe that female resin, once removed from the flower, becomes a crystalline mass within 30 min while male resin remains liquid for weeks. Furthermore, in the Gran Sabana, euglossine bees are seen collecting resin from male flowers shortly after first-light and well before sunrise. Compared to the male flowers, female flowers delay both anthesis and resin secretion by several hours, forcing pollinators to visit males first.

Other possibilities must be considered, for instance, a mechanical or antibacterial synergy between male and female resins which obliges the bee to mix the two. In this case, the insect is forced to visit both sexes in order to obtain some mixture of resins for the required function. This and the previously mentioned scenarios all generate testable predictions of pollinator behavior. Field studies of pollinator visitation patterns to *C. grandiflora* are continuing and will hopefully provide additional insight into this unusual sexually dimorphic pollinator reward system.

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