



The chemistry of an unusual seed dispersal mutualism: bees use a complex set of olfactory cues to find their partner



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Many insect groups are important mutualistic partners of plants. Bees in particular provide an essential mutualistic service to plants: pollination of their flowers. They can also act as seed dispersers for plants, a rare seed dispersal mutualism termed melittochory. One group of known bee seed dispersers are stingless bees (Apidae: Meliponini). Australian stingless bees collect resin from fruits of the eucalypt tree *Corymbia torelliana* (Myrtaceae). In doing so, they disperse the trees' seeds. Therefore, the resin chemistry of *C. torelliana* may be particularly well adapted to attract the bees. While the importance of visual and olfactory cues has been frequently studied in pollination mutualisms, we here provide the first study to investigate the importance of resin compounds as olfactory cues in a bee seed dispersal mutualism. To investigate whether the seed-dispersing Australian stingless bee species *Tetragonula carbonaria* relies on olfactory cues when searching for *C. torelliana* resin and which compounds of the blend they use to identify it, we performed several choice tests with pure resin extracts, fractionated extracts, extracts enriched with *C. torelliana* resin compounds and with single compounds. Olfactory cues were sufficient to attract bees, confirming the importance of olfactory cues for resin-searching stingless bees. However, bees only landed on filter papers with pure resin extract, but not on fractionated or enriched extracts, nor on single compounds. This finding indicates that, instead of relying on just a few specific volatile compounds, the bees used a complex blend as recognition cue including relative compound proportions. Moreover, they showed little tolerance for even slight concentration changes of single components. Such high cue specificity is rather unusual for insects seeking resources, but does, in the case of Australian stingless bees, ensure detection and identification of the resin of their mutualistic partner.

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Many insect species are important pollinators for plants that are obligatorily or facultatively pollinated by animals (Ollerton, Winfree, & Tarrant, 2011). Among these, bees represent the largest group of insect pollinators (Neff & Simpson, 1993; Waser & Ollerton, 2006). Bees can also serve as mutualistic partners of plants by dispersing their seeds, although this is much less studied. This type of mutualism is called melittochory (Wallace & Trueman, 1995) and has been found in three rainforest species: stingless bees dispersing seeds of *Corymbia torelliana* (Myrtaceae) in Australia (Wallace, Howell, & Lee, 2008; Wallace & Lee, 2010; Wallace & Trueman, 1995), *Coussapoa asperifolia magnifolia* (Urticaceae) in

Brazil (Nunez et al., 2008) and *Zygia racemosa* in the Central Amazon (Bacelar-Lima et al., 2006). In all cases, bees are attracted by resin that is produced in the trees' fruits. When the bees collect the resin inside the fruits, the seeds get attached to their legs and are subsequently taken back to their nests, where the resin is removed and the seeds discarded outside (Wallace & Trueman, 1995). In doing so, the bees facilitate seed dispersal at distances of up to 1 km (Wallace et al., 2008).

Resin is produced by plants to seal wounds and deter pathogens and herbivores (Langenheim, 2003), but stingless bees are not affected by the defensive, and for many insect taxa toxic, secretion and are able to use it for nest construction (Leonhardt & Blüthgen, 2009; Roubik, 1989, 2006) and defence (Duangphakdee, Koeniger, Deowanish, Hepburn, & Wongsiri, 2009; Greco et al., 2010; Khoo & Yong, 1987; Leonhardt & Blüthgen, 2009; Roubik, 2006; Velikova, Bankova, Marcucci, Tsvetkova, & Kujumgiev, 2000;

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Velikova, Bankova, Tsvetkova, Kujumgiev, & Marcucci, 2000). Moreover, several stingless bee species incorporate compounds from plant resins in their cuticular surface profiles (Leonhardt, Blüthgen, & Schmitt, 2009; Leonhardt, Rasmussen, & Schmitt, 2013; Leonhardt, Schmitt, & Blüthgen, 2011). These cuticular compounds derived from resin can reduce interspecific aggression (Leonhardt, Jung, Schmitt, & Blüthgen, 2010) and protect their bearers against predators (Lehmberg, Dworschak, & Blüthgen, 2008; Wenzel, 2011). Given the variety of functions that resin fulfils in the bees' ecology, it is a crucial resource limiting the growth and wellbeing of stingless bee colonies (Howard, 1985). Their ability to utilize resin may even have facilitated their diversification in tropical ecosystems (Litman, Danforth, Eardley, & Praz, 2011; Roubik, 1989).

Several plants have evolved resin rewards to attract bees. For example the genera *Dalechampia* (Euphorbiaceae) and *Clusia* (Guttiferae) have replaced the typical pollinator rewards (i.e. pollen and nectar) by resin (Armbruster, 1984).

Chemically, resin is a highly complex blend that can consist of many structurally different compound groups (Langenheim, 2003). Their type and composition depend on the plant genus/family (Langenheim, 2003). Many of these compounds are volatile (Gershenson & Dudareva, 2007; Langenheim, 2003) and resin-seeking insects use them as olfactory cues to detect resin from great distances. For instance, stingless bees in Borneo rely on a blend of volatile mono- and sesquiterpenes to locate resin sources (Leonhardt, Zeilhofer, & Schmitt, 2010). Volatile terpenes are also used by the bark beetle *Dendroctonus ponderosae* to locate their host trees (reviewed by Phillips & Croteau, 1999) and (among other compounds) by honeybees, *Apis mellifera*, to recognize oilseed rape, *Brassica napus*, flowers (Blight et al., 1997) and snapdragon, *Antirrhinum* spp., flowers (Wright, Lutmerding, Dudareva, & Smith, 2005). Plant volatiles can thus play a crucial role in mediating mutualistic and antagonistic interactions between plants and insects. However, although several studies have assessed the importance of olfactory cues in pollination mutualisms (Dudareva & Pichersky, 2006; Knudsen, Eriksson, Gershenson, & Stahl, 2006; Knudsen, Tollsten, & Bergström, 1993; Pham-Delègue, Masson, Etievant, & Azar, 1986), so far no study has investigated the importance of resin volatiles as olfactory cues in seed dispersal mutualisms.

In Australia, beekeepers of stingless bee colonies have reported that bees preferentially collect resin from *C. torelliana* capsules during its fruiting season between December and February, while apparently neglecting most other resin sources and even pollen or nectar foraging (Klump, 2007). The chemistry of *C. torelliana* fruit resin comprises at least 58 compounds, including terpenes, flavonoids and phloroglucinols (Leonhardt, Wallace, & Schmitt, 2011; Massaro et al., 2014). It produces a smell that appears very strong and unique to the human nose (personal observation). *Corymbia torelliana* capsule resin further differs qualitatively and quantitatively from the trunk resins typically collected by stingless bees in Eastern Australia including other Myrtaceae (S. D. Leonhardt, personal observation).

We tested whether stingless bees preferred *C. torelliana* resin over resins from other tree species by offering colonies of *T. carbonaria* a choice of different resins from several plant species. In addition, by replacing the *C. torelliana* capsule resin by its hexane extract, we investigated whether the bees rely on olfactory cues to identify their preferred resin. Finally, to determine whether the bees used the entire resin profile or several of its key compounds to detect *C. torelliana* resin, we performed several choice tests with various extracts, offering pure resin extracts as well as fractionated extracts, extracts modified by adding *C. torelliana* resin compounds and single terpenes.

We hypothesized that, similar to resin-foraging bees in Borneo, *T. carbonaria* foragers are able to detect the resin based on olfactory cues alone. We further predicted that they use a combination of several specific volatile key compounds to identify their preferred resin source.

METHODS

Study Site, Species and Resin Sampling

The study was conducted in southeast Queensland, Australia, between January and April 2013. Behavioural experiments were performed with three hives of *T. carbonaria*, one of the most common species in our study area (Dollin, Dollin, & Sakagami, 1997; Walker, 2010). Bees of this species collect large quantities of resin from various plant species (Leonhardt, Heard, & Wallace, 2014).

Resin for the behavioural experiments was obtained from four different plant species belonging to three different families, all of which are known to be visited by *T. carbonaria* for resin collection (Leonhardt, Wallace, et al., 2011; Wallace & Lee, 2010): *C. torelliana* (Myrtaceae), *Corymbia citriodora* subsp. *variegata* (Ccv) (Myrtaceae), a *Pinus* hybrid (*Pinus caribaea* × *Pinus elliottii*, Pinaceae) and *Araucaria cunninghamii* (Araucariaceae). *Corymbia torelliana* resin was obtained from resin storages of different *T. carbonaria* colonies, because it is produced in minute quantities in the trees' seed capsules (Wallace et al., 2008) rendering it difficult to obtain sufficient amounts directly from the tree. As the trees were fruiting prior to and during the study period, *T. carbonaria* colonies were collecting and storing large quantities of *C. torelliana* resin in their nests. This resin can be easily recognized by colour, smell and the presence of seeds (Massaro et al., 2014). It shows high overlap with resin obtained directly from the tree's fruits and does not contain any wax-derived compounds (Massaro et al., 2014). To ensure that the resin obtained from colonies in our study was also pure and therefore free of hive-derived, this is to say beeswax-derived, compounds, we analysed its chemical composition and compared it with resin obtained from seed capsules using gas chromatography coupled to mass spectrometry (GC-MS) as described in Leonhardt, Wallace, et al. (2011; see Appendix Fig. A1). For a detailed list of compounds found in *C. torelliana* fruit and hive resin see Massaro et al. (2014) and Leonhardt, Wallace, et al. (2011). Resin of all other trees was collected by wounding the tree's trunk with a small knife and attaching a clean clear plastic bag beneath the wound to capture all resin secreted in the following 5–20 h.

Extract Preparation

Freshly collected resins were transferred to the laboratory and kept in a freezer (−18 °C) until extract preparation. For extraction, 0.8–0.9 g resin was weighed into clean 12 ml glass vials, dissolved in 10 ml of hexane, thoroughly shaken for 1–2 min and kept at 5 °C for at least 24 h.

Extract Fractionation

We performed two types of fractionation to examine whether polar or nonpolar compounds were sufficient to attract bees to extracts. First, we used a small silica gel column packed with 500 mg of SiOH (CHROMABOND, 5 ml, Macherey-Nagel, Düren, Germany). We conditioned this column with two column equivalents (CE) of hexane before adding 1.5 ml of pure *C. torelliana* resin extract. The column was then eluted with 3 CE of hexane and the

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