



# *Formica francoeuri* responds to pheromones and defensive chemical cues of social bees

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## Abstract

Insects must detect and respond to diverse chemical cues in a complex chemical landscape. Intraspecific and intraguild communication have received a lot of attention, especially among bee taxa. However, little is known about interspecific chemical communication in general and in non-bees in particular. Ants, such as *Formica francoeuri*, also are opportunistic floral visitors who seek the carbohydrate-rich rewards of flowers. Thus, these ants likely encounter aggressive competitors for nectar, such as bees. Here, we performed a series of experiments to examine how ant foragers respond to bee pheromones, venom and sting glands in the context of foraging. Understanding how ants utilize non-target chemical cues will identify previously cryptic interactions and provide new insights into the hidden mechanisms governing foraging decisions. As the foraging decisions by floral visitors have a direct impact on the pollination mutualism, studies such as this will help us build better predictive models of plant–pollinator networks and develop hypotheses as to how these interaction networks may change with shifts in the floral visitor community.

**Keywords** Chemosensory · Foraging · Formicidae · Avoidance · Grooming · Olfaction · Interspecific communication

## Introduction

Each day, organisms encounter a tremendous array of signals, and decoding the information is likely to be beneficial in many ways. These signals might provide information to enhance foraging efficiency, help an individual find a mate, avoid predators or competitors, or detect toxins (Ha and Smith 2009). Providing the ability to detect many such signals, chemical communication systems are widespread and diverse in insects (Saleh et al. 2007). We expect each insect to experience a diversity of chemical signals, both from their own species and from other species. How does this vital olfactory sense, which is quite well studied in insects (Ali and Morgan 1990; Richard and Hunt 2013; Leonhardt

et al. 2016), shape how organisms interact with their environment? While we know that chemosensation is used for intraspecific communication and plays a role in many of the vital behaviors mentioned above, many questions remain about how interspecific signals are sensed and interpreted.

Intraspecific chemical communication has been well studied in insects, particularly in the context of reproduction (e.g., Leibensperger et al. 1985, Greenberg et al. 2007, and Krupp et al. 2008) and social group coordination (reviewed by van Zweden and d’Ettorre 2010 and Leonhardt et al. 2016). The ubiquity of chemical communication in insects is in part due to the fact that cuticular hydrocarbons (CHCs) cover the cuticles of most insect species and have been coopted to serve a secondary function as a means of chemical communication (Howard and Blomquist 2005). This has been well documented in social Hymenoptera (Blomquist and Bagnères 2010), where CHC profiles can indicate nest-mate status and dominance rank in ants, bees and wasps (e.g., Châline et al. 2005 and Witjes and Eltz 2009). Furthermore, active chemical communication via pheromones is extensively employed across the social insects in a diversity of contexts (Leonhardt et al. 2016), including foraging (Czaczkes et al. 2015), defense (Leonhardt 2017), and avoidance of social conflict (Le Conte and Hefetz 2008).

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In addition to utilizing their own scent to mediate intraspecific interactions and behaviors, insects encounter a complex landscape of odors produced or deposited by other species. Chemically-mediated interspecific interactions have been studied in some specific contexts, including in insect/plant mutualisms (e.g., Raguso 2004), parasitism (e.g., Lenoir et al. 2001), and as a means for predators to detect specific prey (e.g., Allan et al. 1996). We know far less about whether and how insects respond to non-target cues from heterospecific species, particularly in interactions that have not been shaped by coevolution.

With increased attention on the pollinator crisis (Marshall et al. 2019), there has been renewed interest in any interaction at the floral interface that may interfere with floral visitation by pollinators, subsequent foraging for resources, and pollination services (Hanna et al. 2015; Sinu et al. 2017; Barônio and Del-Claro 2018). As part of their normal foraging, many ant species, including our focal

species *Formica francoeuri*, visit plants (Fig. 1). They seek the carbohydrate-rich rewards offered by flowers, extrafloral nectaries, and phloem-feeding insects (Robinson 2005; Wagner and Fleur Nicklen 2010). As a shared resource, flowers are a likely location for ants to encounter bees. Chemical cues of bees, such as components of venom, may be encountered by ants in a variety of contexts. Honey bees, like most social Hymenoptera, will spread venom both on an individual insect's cuticle and the nest surface (Baracchi & Tragust 2017). Venom compounds are applied on the cuticle during self-grooming movements (Baracchi et al. 2011), which has been suggested to serve as protection against pathogens in ants, bees and wasps (reviewed in Fratini et al. 2017). Moreover, venom may be encountered during agonistic ant-bee interactions, as well as in traces detected on floral substrates after grooming or aggressive behaviors by bees.

The detection of bee-associated chemicals or pheromones may allow ants to avoid potential competitors or avoid



**Fig. 1** *Formica francoeuri* foraging (a) in flowers of California fuchsia, *Epilobium canum*, (b) in flowers of hairy yerba santa, *Eriodictyon trichocalyx*, (c) alongside a bumble bee, and (d) on a live honey bee

flowers that have already been drained of nectar. Examining how ants respond to the chemical signals or compounds of other species provides new insights into the factors that influence foraging behavior. In recent years, there has been a lot of interest in mechanisms underlying bee avoidance of ants at floral resources (e.g., Ballantyne and Willmer 2012, and Sidhu and Wilson Rankin 2016). However, little is known about how ants respond to bee-derived chemical compounds and whether ants respond differently to heterospecific cues from a functionally different species with which they lack a shared evolutionary history of interaction. It is important to address these knowledge gaps to recognize the sometimes cryptic interactions that underlie foraging decisions of floral visitors. Here, we performed a series of experiments to examine how ant foragers respond to bee pheromones, venom and sting glands in a foraging context. Understanding these interactions among floral visitors, including nectar thieves and pollinators, may ultimately help us build better predictive models of plant–pollinator mutualism networks.

## Materials and methods

### Study organism

We investigated foraging behavior of *Formica francoeuri* (Hymenoptera: Formicidae), a California native ant. This species is distributed from San Francisco, California to northern Baja California (antweb.org), where it can be found in riparian areas, chaparral, and sand dunes. This species is omnivorous, scavenging dead or moribund insects, including bees, and visiting plants to collect sugars through floral visitation and aphid tending (Fig. 1).

### General experimental setup: lab trials

We used a series of choice tests to assess how *F. francoeuri* responded to chemical cues associated with other flower-visiting Hymenoptera. Foragers were collected from a wild population along the Santa Ana River Trail in Riverside, CA. In the lab, we established microcolonies, each comprising 20–50 foragers, maintained at 25 °C and provided with water and 2 M sucrose solution. We prepared shallow, square Petri dishes and placed 1-cm wide strips of filter paper to section off two opposite corners of the Petri dish (Fig. S1). Approximately, 100 µL of a 2 M sucrose solution was then pipetted into the sectioned off corners. Under a fume hood, the test solution of one of five chemical treatments was applied to one filter paper strip and a control solution was applied to the second filter strip. Treatments tested were: (1) honey bee alarm pheromone ( $N=21$ ), (2) honey bee venom sac ( $N=16$ ), (3) honey bee sting bulb ( $N=12$ ), (4) honey

bee sting bulb and venom sac ( $N=17$ ), or (5) bumble bee venom sac ( $N=27$ ). The ant was placed in the middle of the Petri dish and allowed to choose which strip to cross to gain access to the sucrose solution in 30-min trials. To prevent any cross-contamination between the trials, a new Petri dish and a new ant were used for each trial. No ants participated in more than one trial.

### Response to synthetic honey bee alarm pheromone: isopentyl acetate (IPA)

To test the response of *F. francoeuri* to honey bee alarm pheromone, we distributed 5 µL of 100% isopentyl acetate (Sigma-Aldrich), which is estimated to be 5–8 bee equivalents using the calculations of Rossi et al. (2018), in 1 µL aliquots across the filter paper strip. Thus, when an ant encountered one of the aliquots, it was exposed to 1–1.6 bee equivalents. Five microliters of water was distributed in the same manner for the control. Each trial lasted 30 min, during which we noted the time and duration of each feeding event, which sucrose droplet was visited, and what kind of behavior was exhibited when the ant came in contact with the two filter papers. During this experiment, we noticed the ants engaged in grooming activity after coming in contact with either filter paper.

### Response to honey bee venom sacs, sting bulbs, and a combination of both

We freeze-killed honey bees and kept them at – 80 °C until dissection. Using forceps, we removed the honey bee venom sac and sting bulb. Five microliters of water was used for the control as above. This experiment had three separate experimental treatments: sting bulb on its own (plus any other associated glands within the sting chamber), venom sac on its own, or sting bulb and venom sac. The venom sacs and sting glands were removed and then put into clean glass vials on ice to be kept fresh until they were needed in trials. Rather than pipetting the treatment, the individual venom sacs, sting bulb, or both a venom sac and sting bulb were ruptured onto the filter paper directly. Data collection followed the same protocol as described above with addition that we noted the time, duration, and location of any grooming activity.

### Response to bumble bee venom sacs

To determine if any comparisons could be made between the bumblebee venom and the honey bee venom and their impact on ant behavior, we performed micro-dissections to harvest the venom sacs of lab-reared *Bombus impatiens*. Bumblebees were freeze-killed and retained at – 80 °C until dissection to keep the venom sacs intact. Using forceps,

we pulled the stinger out and exposed the distinctive pear-shaped venom sac. Handling of the venom sac and data collection followed the same protocol as previously described for honey bee venom sacs.

### Field trials: response to isopentyl acetate

We carried out a field experiment to examine the behavioral response of *F. francoeuri* foragers to synthetic honey bee alarm pheromone (100% isopentyl acetate) compared to other toxic and/or volatile synthetic chemicals (100% ethanol and 5% ethyl butyrate). Ethyl butyrate is a food additive with a pineapple-like smell which is used in olfactory experiments in a diversity of animal taxa (e.g., Goldsmith and Goldsmith 1982, Cunningham et al. 2016, and Lee et al. 2017). Due to the lower detection threshold of ethyl butyrate in comparison to isopentyl acetate and ethanol (Salo 1970; Takeoka et al. 1989), we used a 5% dilution of ethyl butyrate in the field experiments, a concentration in the range of that used in other studies (Goldsmith and Goldsmith 1982; Cunningham et al. 2016; Stuhl 2020). We carried out this experiment over the course of two mornings in May, 2020, along the middle fork of Lytle Creek in the San Gabriel mountains of southern California. The *F. francoeuri* colony density is relatively high in this area, and the ants tend to nest at the base of shrubs in the creek floodplain and along a forest service road cut. We identified individual plants of either *Salvia apiana* or *Eriodictyon trichocalyx*, with *F. francoeuri* workers actively foraging on multiple stems, and we applied each treatment to a separate stem with a similar level of pre-application activity. We alternated the order of application of chemicals on each plant to ensure that the order of application would not influence the response of the ants. Each application was observed for 3 min, with a break of at least three additional minutes between treatments on the same plant. Due to the high ambient temperatures and very low ambient humidity, we applied 100  $\mu$ L of the focal chemical to ensure the chemical would remain throughout the entire observation period. We applied each chemical to a separate stem below the area where ants were actively tending aphids or foraging for nectar and filmed the behavioral response for 3 min after application. The experiment was replicated ten times. We manually scored the videos, noting the number of times that a worker completely crossed the application area, contacted the area and turned around, self-groomed, and interacted with other workers (distinguishing antennation, trophallaxis, and aggression). We scored the response for both one and 3 min to ensure that any observed differences did not result from different evaporation rates of the three compounds. Trials with water as the control had to be excluded from analysis because the workers would cease other activities to collect the water droplets.

### Statistical analyses

We used generalized linear mixed models (GLMM) to assess if the number of behavioral events is affected by bee chemical cues using the package “glmmTMB” (Brooks et al. 2017). We tested whether the frequency of behavioral events (feeding or grooming) per trial was influenced by treatment (control or chemical cue) using a GLMM with a Poisson error structure, with trial as a random effect. A feeding event occurred when an ant opened its mandibles and fed for > 1 s, while a grooming event occurred when an ant engaged in self-grooming for > 1 s while in contact with either the control or experimental filter paper. To assess the duration of grooming per trial, we used a GLMM with a negative binomial error structure where grooming duration was the response variable, treatment (control or chemical cue) was the fixed effect, and trial was the random effect. IPA experiments were excluded from all grooming analyses because there were too few visits to the treatment droplet. Grooming events were counted when grooming was conducted on the filter paper or if ant crossed the filter paper immediately prior to grooming. The function, `romr.fnc`, from the package “LMERConvenienceFunctions” (Tremblay and Ransijn 2015) was used to evaluate extreme outliers. Following the recommendations of Zuur et al. (2009), we removed one to two extreme outliers in four models. Effect sizes were determined using the function, `cohen.d`, in “effsize” (Torchiano 2018). Following Cohen (1998), effect sizes of 0.2 were considered small, 0.5 were considered medium and greater than 0.8 were considered large. For every analysis, we report the parameter estimate for treatment and 95% confidence intervals (Table 1). We excluded from analysis any trial in which the ant did not engage in at least one feeding event.

To compare the response of workers in the field to IPA, ethyl butyrate, and ethanol, we constructed five linear mixed effects models with the number of ants performing each behavior (crossing the application area, encountering and turning back from the area, self-grooming, antennating, and engaging in aggressive interactions) for 3 min following chemical application as the response variable in each respective model, treatment compound as the explanatory variable, and plant as a random effect to account for the blocked experimental design and the observation that plants varied substantially in baseline ant activity. The mixed effects models were implemented using the `lmer` function in the “lme4” package (Bates et al. 2015), and post hoc tests were carried out with the `lsmeans` function (“lsmeans” package, Lenth 2016). We repeated these analyses for behavioral scores of the first minute only. All analyses were conducted in R v. 3.6.1 (R Core Team 2020).

**Table 1** Parameter estimates for treatment variable and 95% confidence intervals for all analyses from generalized linear models (GLMs)

	Variable estimate	95% CI	Cohen's <i>d</i>	<i>P</i> value	
<i>Apis</i> Isopentyl acetate (IPA) ( <i>N</i> =21)					
Frequency of feeding events	- 1.4663	[- 2.065, - 0.934]	-0.80	<0.0001	***
<i>Apis</i> venom sac ( <i>N</i> =16)					
Frequency of feeding events	- 0.3637	[- 0.097, 0.843]	-0.25	0.13	
Frequency of grooming events	0.5232	[0.092,0.970]	0.78	0.019	*
Avg duration of a grooming event (in s)	1.9345	[1.7666, 2.1097]	1.40	<0.0001	***
Total duration of all grooming events (in s)	1.802	[1.7039, 1.9025]	1.42	<0.0001	***
<i>Apis</i> sting gland ( <i>N</i> =12)					
Frequency of feeding events	- 0.3994	[- 0.6972, - 0.0861]	- 0.38	0.010	**
Frequency of grooming events	0.7985	[0.258, 1.378]	1.18	0.0049	**
Avg duration of a grooming event (in s)	2.5	[2.2843, 2.7297]	1.48	<0.0001	***
Total duration of all grooming events (in s)	3.4138	[3.2035, 3.6388]	1.29	<0.0001	***
<i>Apis</i> venom sac and sting gland ( <i>N</i> =17)					
Frequency of feeding events	- 0.5790	[- 1.079, - 0.0543]	- 0.44	0.035	*
Frequency of grooming events	0.4140	[0.0143, 0.8269]	0.58	0.045	*
Avg duration of a grooming event (in s)	0.1146	[0.0146, 0.2149]	0.11	0.0248	*
Total duration of all grooming events (in s)	0.5320	[0.4674, 0.597]	0.33	<0.0001	***
<i>Bombus</i> venom sac ( <i>N</i> =27)					
Frequency of feeding events	- 0.0572	[- 0.113, - 0.002]	- 0.53	0.04	*
Frequency of grooming events	0.4928	[0.377, 0.610]	0.59	<0.0001	***
Avg duration of a grooming event (in s)	- 0.0572	[- 0.113, 0.0019]	- 0.19	0.05	
Total duration of all grooming events (in s)	0.4666	[0.443, 0.490]	0.62	<0.0001	***

If the confidence intervals of the treatment parameter do not bound zero, then treatment is significant. Cohen's *d* reports the effect size with Hedges' correction. When *d* < 0, treatment decreased the mean response; when *d* > 0, treatment increased the mean response. \*Indicates *p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001

## Results

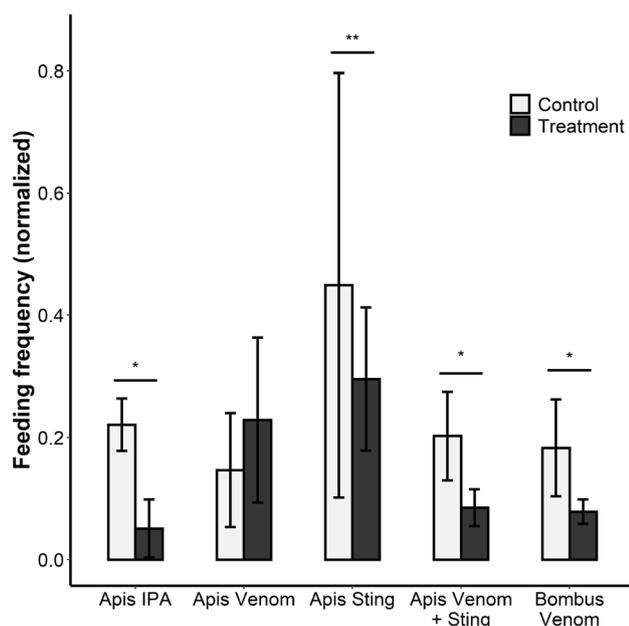
Overall, *Formica francoeuri* ants preferentially fed on the control droplet for all lab assays. This indicated a medium to strong avoidance of the treatments in most contexts (Fig. 2: *Apis* IPA: *Z*=5.12, *p*<0.0001, *d*= - 0.8; *Apis* sting: *Z*=2.57, *p*=0.01, *d*= - 0.38; *Apis* venom + sting: *Z*=2.11, *p*=0.035, *d*= - 0.44; *Bombus* venom: *Z*=2.03, *p*=0.04, *d*= - 0.53). We did not detect an effect of *Apis* venom on ant feeding behavior (*Z*= 1.52, *p*=0.13).

The frequency of grooming behavior per trial in lab assays was much higher after coming into contact with the chemical cues as compared to the control filter paper (Fig. 3a: *Apis* Venom: *Z*=2.35, *p*=0.019; *Apis* sting: *Z*=2.813, *p*=0.0049; *Apis* venom + sting: *Z*=2.00, *p*=0.045; *Bombus* venom: *Z*=8.28, *p*<0.0001). Ants not only groomed more after contacting defensive compounds of bees, but they spent significantly more time grooming after contacting the treatment filter papers than the control filter papers. Ants spent 12% more time grooming per grooming event after contacting *Apis* venom + sting than the control filter paper (*Z*=2.25, *p*=0.025, *d*=0.11). The average duration of a *F. francoeuri* grooming event was nearly seven times higher

for honey bee venom than the control (Fig. 3b: *Z*=22.12, *p*<0.0001, *d*= 1.40). This increased grooming response was even more extreme for the sting gland (Fig. 3b: *Z*=22.03, *p*<0.0001, *d*= 1.48).

The total time ants spent grooming on a per trial basis was also higher for all chemical cues in lab assays (Table 1; Fig. 3c). Ants spent nearly 70% longer grooming after contacting *Bombus* venom sacs and sixfold more time grooming after contacting *Apis* venom sacs (*Z*<sub>Bombus</sub>=39.04, *p*<0.0001, *d*=0.62; *Z*<sub>Apis</sub>=35.57, *p*<0.0001, *d*=1.42). Strong effects on grooming were observed after contacting the *Apis* sting gland, where ants spent 30-fold more time grooming than after contacting the control filter papers (*Z*=30.79, *p*<0.0001, *d*=1.29). We observed the same trend for the combination of the honey bee venom sac and sting glands, where ants spent 61% more time grooming on the chemical cue than control filter paper (*Z*=16.1, *p*<0.0001, *d*=0.33).

The field experiment revealed that foraging ants differed in their response to synthetic honey bee alarm pheromone compared to another volatile chemical that is not biologically relevant (ethyl butyrate) and an ethanol control (Fig. 4). In our comparisons of these three treatments administered



**Fig. 2** Feeding frequency in bioassays (normalized number of feeding events per trial). *Formica francoeuri* fed more frequently on the control than the treatment for most of the experiments. Data were normalized, rescaled to range between 0 and 1. IPA: isopentyl acetate. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

on the same plant, we observed that foragers were more likely to turn around (Fig. 4b:  $F_{2,18} = 8.99$ ,  $p = 0.0020$ ), to self-groom (Fig. 4c:  $F_{2,18} = 10.24$ ,  $p = 0.0005$ ), or to interact aggressively (Fig. 4d:  $F_{2,18} = 5.50$ ,  $p = 0.0137$ ) after encountering IPA compared to the other compounds. In contrast, we found no significant pattern in the number of ants crossing the chemical application site ( $F_{2,18} = 1.91$ ,  $p = 0.177$ ) or in the number of antennations ( $F_{2,18} = 1.96$ ,  $p = 0.169$ ). We observed the same pattern when scoring behaviors for only the first minute after chemical application (Fig. S2).

## Discussion

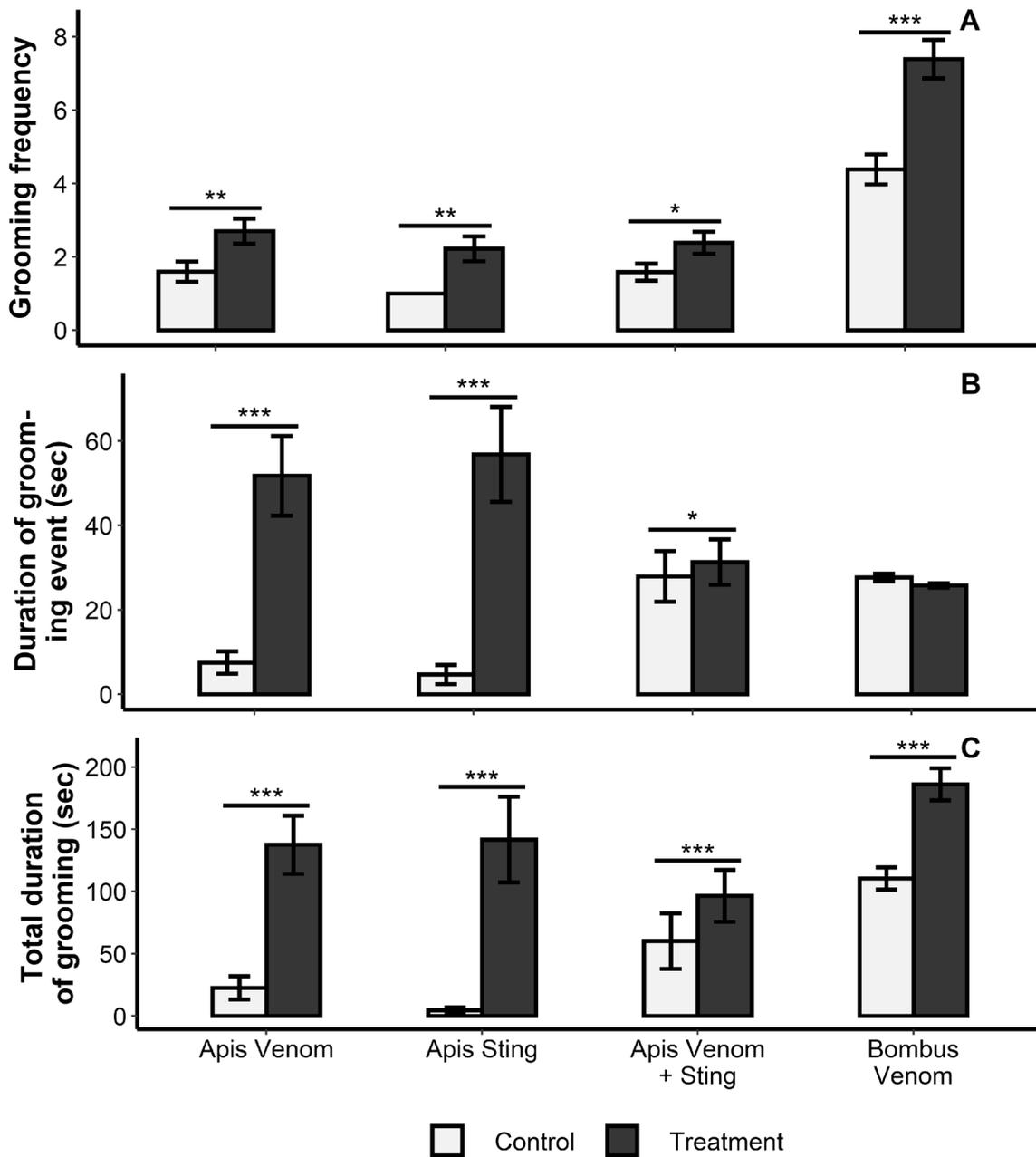
While *Formica* spp. have a generalized diet of arthropod prey and sugars derived from aphid honeydew and nectar (Sorvari et al. 2008; Iakovlev et al. 2017; Rosumek et al. 2017), little is known about their interactions with non-ant competitors, such as bees. Here, we document the behavioral response of *Formica francoeuri* to chemicals associated with bee defense. The ants clearly preferred feeding from the control solution and exhibited significantly lower feeding rates after contacting nearly all *Bombus* and *Apis*-derived chemicals tested. *Formica* ants may be responding similarly to the two bee venoms in part because 72% of bumblebee venom compounds have a honeybee venom homolog (Van Vaerenbergh et al. 2015). In the field, we noted a clear

increase in activity in response to IPA application along a foraging path, with ants tending to turn back from the part of the stem where the compound was applied and, on some plants, increase the frequency of self-grooming and agonistic interactions.

Chemical cues mediate behavioral responses in many ant species (Knaden and Graham 2016). Ants rely heavily on chemical cues while foraging to navigate (Knaden and Graham 2016; Freas and Schultheiss 2018), to locate food resources (Buehlmann et al. 2014; Nelson et al. 2019) or aphid partners (Verheggen et al. 2012), as well as to recruit nestmates to resources (Fourcassié et al. 2010; Wust and Menzel 2017). Moreover, some ants use chemical cues to minimize risk, for example, responding to the release of alarm pheromone by nestmates and helping defend (Mizunami et al. 2010) or rescue nestmates from an enemy threat (Hollis 2017). While we know far less about how ants respond to heterospecific chemical cues, behavioral avoidance of such cues appears to be common. For instance, some ants will avoid the olfactory cues associated with competitor species (Wust and Menzel 2017) or predatory spiders (e.g., Bucher et al. 2015).

The ability to detect the presence of competitors and minimize the risk of competition may facilitate coexistence among species with overlapping resource use (Camarota et al. 2018). Such traits would be beneficial in both the contexts of exploitative and interference competition, particularly as the bee-derived chemicals being tested are all associated with defense and are released during agonistic interactions. Here, we documented how a native ant (*Formica francoeuri*) detected chemical traces of heterospecific floral visitors and adjusted their foraging behavior in response. Consistent with this hypothesis, the ants preferred feeding on control solution (irrespective of what bee defensive chemical was tested).

Bee venom is most commonly thought of as the result of a sting. However, bees, like most social insects, will spread venom across their cuticle and the nest surface (Baracchi and Tragust 2017). Thus, the presence of these bee-derived defensive chemicals at floral resources not only would indicate the presence of a potential competitor but could also provide information that agonistic or aggressive interactions have occurred. Moreover, other hymenopteran venoms or their constituents are known to elicit avoidance by ants (Blum et al. 1991) and wasps at high concentrations (Weston et al. 1997). While perhaps not common, bumblebees have been known to bite and engage in aggressive behaviors towards ants (Miner 2018). Thus, ants may actually be minimizing potential competition for nectar and decreasing the likelihood of aggressive interactions by avoiding floral resources with bee-derived defensive compounds. Further research is needed to examine whether ant responses are actively decreasing the pressures of interference and



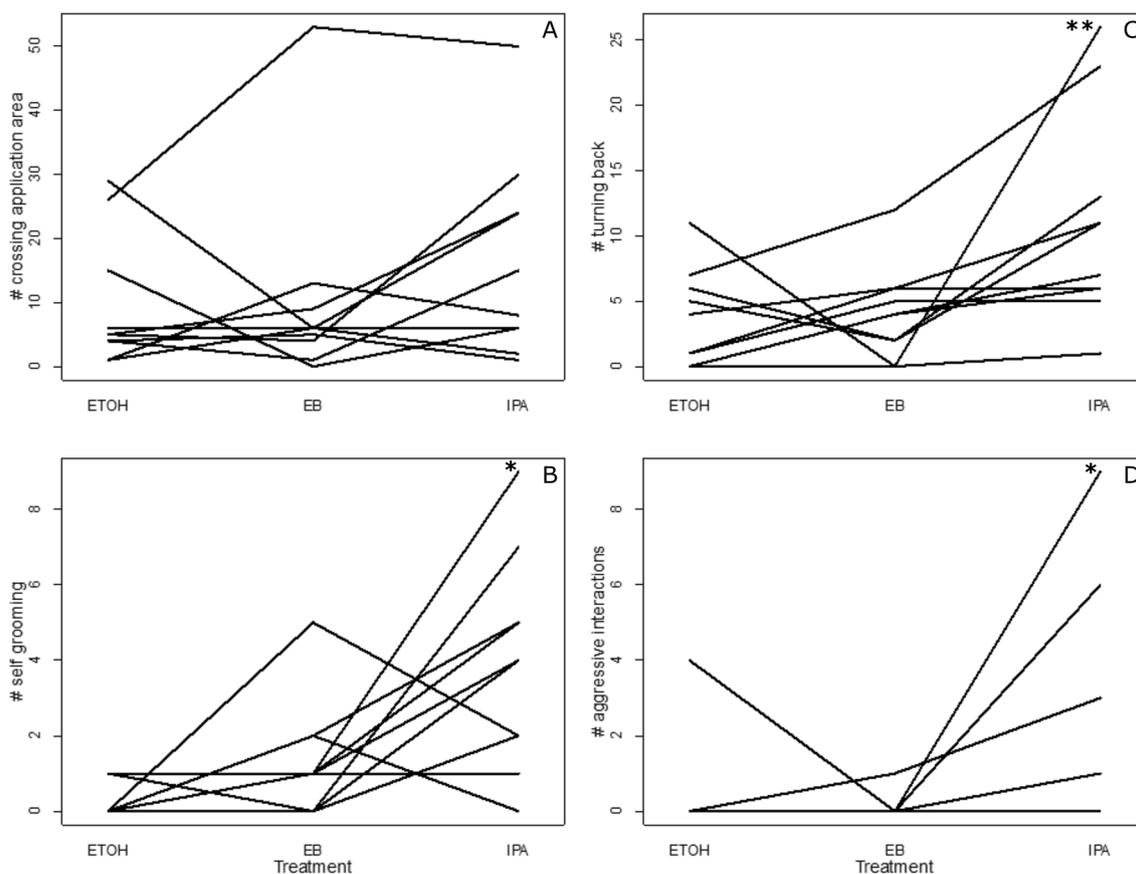
**Fig. 3** Grooming behavior in bioassays. **a** *Formica francoeuri* groomed very frequently after coming into contact with *Apis mellifera* and *Bombus impatiens* chemical cues. **b** The average duration of a grooming event by *F. francoeuri* was longer after coming into

contact with bee chemical cues than controls. **c** On a per trial basis, *Formica francoeuri* spends more total time grooming after contacting treatments than control for experiments with *A. mellifera* and *B. impatiens*. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

exploitative competition, and to disentangle these two mechanisms.

In addition to altering their foraging behavior, we observed that ants responded to the bee chemicals by adjusting their grooming behavior both in lab and field assays. A very common animal behavior (Böröczky et al. 2013), insects frequently groom to remove foreign materials from their cuticle (Hlavac 1975). Self-grooming is a critical

mechanism to remove environmental contaminants from the antennae (Böröczky et al. 2013) or distribute products from glands (e.g., the metapleural gland, Fernández-Marín et al. 2006) across the cuticle. A lot of attention has been paid to grooming behavior due to its role in helping to reduce parasite or pathogen exposure (Reber et al. 2011). In this study, self-grooming was almost exclusively observed after contacting noxious chemicals (e.g., bee venom, bee



**Fig. 4** The field experiment revealed a strong response of foraging *Formica francoeuri* workers to the application of synthetic honey bee alarm pheromone (IPA) compared to an ethanol control (ETOH) or an ethyl butyrate control (EB) during 3-min observations. In these interaction plots, each line shows the differences in worker response during 3 min following the application of these compounds to different stems of the same plants. Plants differed in baseline ant activity level, and were included as random effects in each model. We find

that the number of workers crossing the application area did not differ significantly by treatment (**a**), but that instances of self-grooming (**b**), the number of workers that turn back from the application area (**c**), and the number of aggressive interactions (**d**) all increased significantly in the IPA treatment relative to both the ethanol and ethyl butyrate controls (\*indicates the significant treatment in post hoc tests). \* $p < 0.05$ , \*\* $p < 0.01$

pheromones, or ethyl butyrate). Since direct contact with the chemicals occurred immediately before initiation of the grooming behavior by *F. francoeuri*, it is most likely due to the ants attempting to remove the traces of these chemicals from their antennae and cuticle. Removing chemicals from the antennae likely allows ants to continue chemosensation and to maintain the ability to discriminate among environmental stimuli (Crozier and Pamilo 1996; Böröczky et al. 2013; Richard and Hunt 2013). Alternatively or additionally, self-grooming could be employed by ants to rapidly spread their own chemical signals (e.g., alarm pheromone, which is produced in the Dufour's gland of *Formica* species (Lenz et al. 2013)).

Field assays yielded additional insights into how the ants respond in a more natural setting. We still observed increased self-grooming following application of artificial honey bee alarm pheromone compared to the application

of control compounds, suggesting that this response is stronger in the presence of a biologically relevant, hetero-specific chemical cue. We also noted that a large proportion of workers encountering the IPA on the stem of the plant where they are foraging will stop and turn around, rather than crossing. Nevertheless, some workers still cross stems treated with IPA, ethyl butyrate, and ethanol. Intriguingly, we also detected an increase in the rate of agonistic interactions directed against fellow foragers (as well as an aphid and a scale insect in rare cases). Paired with our field observation of *F. francoeuri* capturing and subduing a live honey bee worker (Fig. 1d), we suggest that workers could also respond to bee pheromones and venoms for predatory purposes.

Our discovery that ants actively respond to bee-derived defensive compounds in the context of foraging raises several intriguing questions. First, how long do these non-target

cues affect ants' subsequent foraging decisions? If the effects are only observed in the short term, this could indicate that ants are avoiding direct interactions with bees or avoiding interactions with predators of bees. If the effects are sustained, this could indicate that ants use these heterospecific cues to avoid areas that have already been visited by foragers or that the ants cannot distinguish cues based on their deposition time. Investigating these interactions further in the field could provide some insights into these potential differences. Second, are the responses of ants to these bee-derived chemicals dosage dependent? While we used volumes relevant to roughly one bee equivalent, future research should explicitly quantify the amounts of venom and pheromone deposited incidentally during resource visitation or during an aggressive interaction at a resource and assess whether ant responses shift with chemical concentration. Lastly, the strong and consistent self-grooming response of ants that encounter bee-derived chemicals warrants future investigation. For instance, it would be interesting to observe nestmate interactions following exposure of a forager to bee defensive chemicals. Whether that forager elicits an aggressive response, receives grooming from a nestmate, or elicits no unusual response will shed light on the reasons that ants self-groom in this context. Generally, heterospecific chemical signal responses are overlooked in the literature outside of studies on mutualism or parasitism, but are likely to play a major role in foraging decisions and other key behaviors. Recognizing these cryptic interactions may provide new insights into the dynamic drivers of shifts in floral visitor communities.

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