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The Nasonov gland pheromone as a potential source of death cue in *Apis cerana*

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ABSTRACT

The ability to detect and remove dead adult bees is an essential part of honeybee colony fitness that prevents the spread of pathogens. Fatty acid olfactory cues stimulate undertaking behavior among different social species within Hymenoptera, but the chemicals responsible for the death cue in *Apis cerana* have not yet been identified. We explored the Nasonov gland as a potential source of these chemicals in *A. cerana*. Gas chromatography indicated that unlike *A. mellifera*, the *A. cerana* Nasonov gland does not contain any volatile terpenes, only fatty acids. As a bioassay, dead honeybees were rinsed free of their individual cuticular hydrocarbons via dichloromethane and two concentrations of oleic acid and a synthetic blend of the Nasonov pheromone in *A. cerana* were applied to the dummies. Results showed that oleic acid did not stimulate corpse removal in *A. cerana*. However, the synthetic pheromone blend of *A. cerana* Nasonov did stimulate removal.

1. Introduction

Honeybees live in social colonies containing tens of thousands of members. In such dense living conditions, colonies must protect themselves from the invasion of pathogens. To protect against these threats, one defense is the individual immune system. However, some studies have shown that at the individual level, honeybees invest less genetically in their immune system than other solitary insects (Evans et al., 2006). A second option available to the colony is to mount a collective defense against parasites and pathogens called social immunity (Cremer et al., 2007; Wilson-Rich et al., 2009; Meunier, 2015). One threat is the potential for opportunistic pathogens to enter the colony via dead conspecifics (Cremer et al., 2007). While most honeybees die in the field, workers that die in the nest must be removed (Sun and Zhou, 2018). In honeybees, it is not only adult corpses that must be removed, but also dead or diseased larvae. The detection, uncapping of wax cells, and subsequent removal of larvae that are sick is another social immunity trait in honeybees called hygienic behavior (Mcafee et al., 2018). Table 1.

Wilson coined the term necrophoresis in a pioneering study of ants (1958). In honeybees, this behavior is often called undertaking behavior and is performed by approximately 10% of the workers in the colony that are aged 2–3 weeks old (Trumbo et al., 1997; Visscher, 1983). Honeybee colonies engage in temporal polytheism and have specific work tasks at different ages. Undertaking is similar to guarding behavior at the entrance, which is performed by bees of similar ages, with nearly the same levels of gene expression in the brain (Cash et al., 2005). In other bee species like Bumble bees, it is the guard bees that are the undertakers (Walton et al., 2019). Not enough research has been done on *A. cerana* specifically and currently it has just been assumed that temporal polytheism functions like that of *A. mellifera*. This is an area to be further explored in the future.

Because of the close genetic relatedness and proximity of the *Apis* species, they are vulnerable to the same pathogens, and it is beneficial for the dead to release a removal cue (Meunier, 2015). One study on the ant, *Myrmica rubra*, showed that colony survival was adversely affected when workers were prevented from removing their dead (Diez et al., 2014). In social insects, the corpse induces removal to benefit the fitness of the entire colony (Sun and Zhou, 2013). This is achieved through chemical cues.

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Table 1
The list of codes that represent all of the behaviors that bees displayed towards dummies. Behaviors 1–5 were considered not aggressive and 6–9 were considered aggressive.

Code	Behavior
1	Bumped into or walked over but ignored
2	Encountered and antennated for < 1 s
3	Encountered, antennated briefly, then the bee circled around and returned
4	3 or more bees formed a cluster around the corpse
5	Bees stood on top of the corpse licking it and antennating it for > 1 s.
6	Antennated the corpse then spun around and began vigorously fanning as an attempt to fan the corpse away from the entrance
7	Charged the corpse
8	Drag the corpse with mandibles
9	Removed the corpse from the test area

It's well established that olfactory cues are what stimulate undertaking behavior, and there is no evidence to support a visual cue (Sun et al., 2018). Research has documented the recognition of unsaturated fatty acids as an indicator of dead conspecifics in ants, termites, bees, as well as less social gregarious insects (Wilson et al., 1958; Howard and Tschinkel, 1976; Gordon, 1983; Cremer et al., 2007; Yao et al., 2009; Ulyshen and Shelton, 2011). These chemicals are likely highly conserved across social insect groups and have been called necromones, meaning particular chemicals released after death that modify the behaviors of nest-mates (Yao et al., 2009). The chemical most associated with the death cue across social insect phyla such as ants, termites, and bees is oleic acid (Wilson et al., 1958; Howard and Tschinkel, 1976; Gordon, 1983; Cremer et al., 2007; Yao et al., 2009; Ulyshen and Shelton, 2011; Sun and Zhou, 2013; Mcafee et al., 2018; Sun et al., 2018). A death cue is a post-mortem change to the surface chemicals of a dead body that cause living insects to react in specific ways (Sun et al., 2018).

Context is important in the response that chemicals elicit from insects. Gordon's (1983) work showed that ants will either removal oleic acid treated corpses or bring them into the nest, and that these factors are context dependent. Likewise, in the ant species *Temnothorax rugatulus,* alarm pheromone repels or attracts ants depending on where they are in relation to the nest (Sasaki et al., 2014). It has also been shown that there is a life associated chemical on the Argentine ant, *Linepithema humile,* that masks the removal impulse cue and that these life chemicals, dolichodial and iridomyrmecin, disappear rapidly after death, which causes the actual removal (Choe et al., 2009). Unlike ants, there has not been a clear identification of a "fatty acid death cue" in honeybees (Sun and Zhou, 2013). The odors associated with honeybee hygienic behavior, β-ocimene and oleic acid, have recently been identified (Mcafee et al., 2018). Yet the chemicals involved in the task of undertaking have not been clearly described. Recently however, it has been shown that bees who engage in hygienic behavior are highly likely to remove dead adults from the hive, raising intriguing questions about whether or not there is an overlapping mechanism or death cue (Perez and Johnson, 2019). Still, across the *Apis* family, the association of oleic acid with undertaking has not been confirmed and is contentious (Perez and Johnson, 2019).

Honeybees, ants, and termites all show species-specific necrophoric behavior (Sun and Zhou, 2013). In the case of honeybees in particular, not enough research has been done to make adequate comparisons between species. This makes a separate evaluation of *A. cerana* necrophores is important and novel.

In honeybee nestmate recognition, there is a unique colony level "fingerprint" comprised of cuticular hydrocarbons (CHCs) and fatty acids (Breed, 1998). When bioassays were done looking at nestmate recognition, it was shown that major and minor hydrocarbons did not have a large effect on recognition of individuals (Breed, 1998). Fatty acids on the other hand, had strong results showing that palmitic, oleic, linoleic, and linolenic acids are extremely important in nestmate recognition among honeybees (Breed, 1998). Further work has shown that a change in this fingerprint via a change in acid or hydrocarbon levels, will alert guard bees (Buchwald and Breed, 2005).

Honeybees receive many pheromonal signals inside their dark hives. One of these pheromones is called the Nasonov pheromone and is found in a gland located between the 6th and 7th tergites of the abdomen (Mcindoo, 1914). The chemicals produced by the Nasonov gland in *A. mellifera* have long been identified (Pickett et al., 1980). However, the chemical composition of the *A. cerana* Nasonov gland has not been comprehensively identified. In this study, (1) we analyzed the chemical components of the Nasonov gland in *A. cerana*, (2) performed an analysis on the chemical composition of the bodies of dead worker bees from 0 to 256 h after death, and (3) performed a behavioral assay to test detection and removal of oleic acid or synthetic Nasonov treated dead bees at the entrance. We hypothesized that the chemical cues coming from the bodies of deceased adult *A. cerana* bees could in part be from the Nasonov gland.

2. Materials and methods

2.1. Chemical analysis of the A. cerana Nasonov gland

2.1.1. Pheromone chemical identification

A chemical analysis was done on the *A. cerana* Nasonov gland and also on the species *A. mellifera*, the European honeybee. All of the experimental colonies, bees, and chemicals were provided by the Chinese Academy of Science Xishuangbanna Tropical Botanical Garden and the experiments and analyses were conducted in Kunming, China. The sampling and experimental analysis were conducted between the months of April through October, between the hours of 9 am to 5 pm. Both species of bees were collected from the front entrances of hives using a 5 cm plastic jar with screened holes at the top. The goal was to analyze the glands of freshly killed bees, so bees were first frozen 10 min and then corpses were immediately processed for pheromone extraction using solid phase micro-extraction (SPME). In order to perform this extraction, the venom sac was removed and an elbow tweezers was used to press down on the 6th abdominal segment, exposing the Nasonov gland. After the gland was exposed, the head of the SPME was used to swipe the gland 12–15 times per bee. Bees were sampled from a total of 3 colonies per species, with 7–8 individuals per colony, all undergoing rapid GC analysis.

We used an HP-7890B GC (Agilent, US) with FID for GC-FID analysis, the SPME swiped samples were injected into an HP-5 column (30 m × 320 μm × 0.25 μm, Agilent, US) with N_2 as the carrier gas. The flow rate was 37 cm/s, 250 °C without split injection, SPME analysis for 5 min, the column temperature was maintained at 100 °C for 1 min, and the temperature was raised to 5 °C/min to 280 °C; HP-FFAP capillary columns (30 m × 320 μm × 0.25 μm, Agilent, US) were also used, the analysis conditions were inlet temperature at 240 °C, and the oven was held at 80 °C for 2 min, then the temperature was first increased to 180 °C at 10 °C/min, and then 230 °C, at 5 °C/min for 10 min. The FID detector temperature was set to 250 °C. The data were analyzed using Chemstation (Agilent Technologies, US) software. After every two SPME injections, the oven was warmed to 230 °C and the column was cleaned for 5.5 min. Standards were used to identify chemicals found in the GC analysis.

2.1.2. Pheromone quantification analysis

Each standard was purchased from TCI (Tokyo, Japan). Each group was dispensed into an n-hexane solution with a concentration gradient from 1 ng/μL to 1 μg/μL. An external standard method curve was prepared to quantitatively analyze the absolute content of each active component of each bee.

2.2. Analysis of the chemical profile of worker bee bodies at different time points after death.

To test the changes in body chemicals released after worker bee death, we checked the chemicals released from 0 to 256 h after freezing *A. cerana* bees. Each chemical was then quantitatively analyzed. Bees, chemicals, and laboratory space was provided by the Chemical Ecology Group of the Chinese Academy of Science, Xishuangbanna Tropical Botanical Garden. The experiment was performed in Kunming, China from September to October of 2016. *A. cerana* worker bees were captured at the entrance in the same manner as in the chemical analysis experiment. They were freeze killed at −20 °C for 10 min, and then placed in a 4 ml glass vial at hive temperature for different lengths of time (0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h, 32 h, 64 h, 128 h, 256 h). Then a 65 μm PDMS/DVB non-polar SPME extraction head was inserted into the glass vial, where it was left to absorb any compounds for 30 min. Afterwards a fast action GC quantitative analysis was performed. The SPME extracts were injected into the GC under the following conditions: The chromatographic column was an HP-FFAP capillary column, the inlet temperature was 240 °C, and the oven was held at 80 °C for 2 min. Then, the temperature was increased to 180 °C at 10 °C/min, and then 5 °C/min until it reached 230 °C where it was held for 10 min. The FID detector temperature was 250 °C. Data were analyzed using Chemstation software. In total, we analysised the chemical profile of 5 worker bee bodies at different time points after death.

2.3. Behavioral bioassay

We performed a bioassay to assess the behavioral response to oleic acid and a synthetic chemical blend with identical composition to the Nasonov gland of *A. cerana* . The experiment was conducted from June until October of 2019 in Kunming and Diqing, Yunnan, China. The experiment was conducted between the hours of 9 am to 6 pm, when *A. cerana* heavily uses the entrance.

First, “dummies” were created by collecting 60–90 bees from *A. cerana* colony entrances. Bees have cuticular hydrocarbons (CHCs) that distinguish nestmates from non-nestmates (Singer, 1998). Thus, it is necessary to wash the bees of CHCs and turn the worker bee into a scentless dummy. This method has been used successfully before to remove the CHCs from the bodies of dead hornets (Dong et al. 2018). Each day, the jar containing the bees soaked in dichlorome-thane was shaken for several minutes, and then the liquid was changed. After day three, the bees were removed and rinsed individually in dichloromethane one final time. Then the bees were set out under the sun in a petri dish to dry. Some initial tests were done in order to ascertain if the bees were truly scentless. The untreated dummies were placed at the entrance of the colonies and then observed for any aggressive behavior or removal by the guard bees. This would have indicated that there was still a scent on the body of the bee, triggering defensive behavior. If no such behavior occurred, it was satisfactory that the bees were truly become “dummies” and the experiment could begin.

Two treatments were applied to dummies: pure oleic acid (>99%, Tokyo Chemical Industry Co., Japan) and a synthetic *A. cerana* Nasonov blend. The Nasonov blend was created using the amount per bee found in our chemical analysis (see Table 2). Two oleic acid concentrations, 100% and 10%, were tested based on Mcafee et al. (2018). The Nasonov blend concentrations were 100 bee equivalent and 10 bee equivalent. Control dummies were treated with hexane at the same amount as the other two treatments.

De-scented dummy bees were placed on an extended piece of cardboard, 20 × 10 cm, at the hive entrance. Using a pipette, 10 μl of the particular chemical concentration was placed on the abdomen of the dummy and the behaviors of the bees were observed for 5 min, identifying whether or not bees removed the corpses from the entrance area. Only corpses that were intentionally grabbed by worker bees and flown away from the test area were counted as removed. We also examined the overall behavior of the worker bees towards the treated corpses, using a behavior coding system based on Visscher (1983). Three additional codes were added to describe behaviors not mentioned by Visscher. We classified the behaviors into non-aggressive and aggressive behaviors in the table 1:

In our pre-experiments the bodies were left inside the hive and observed. However, because we were interested to view a range of behaviors exhibited towards the corpses, we found that only having 2 frames inside an observation hive often did not give an adequate representation of typical colony strength. Thus, we moved the experiment to just in front of the entrance of 3 normal sized colonies, to see a better representation of behaviors at normal colony strength. It has been shown that guarding and undertaking are performed by bees with no differences in gene expression in their brains (Cash et al., 2005). Thus, we decided we could get an accurate representation of undertaker behavior at the entrance as well.

In total, we tested three different colonies of *A. cerana*, with 15 dummy tests per chemical and concentration, as well as a control test. We repeated the test 3 times per colony and concentration.

Table 2
The quantitative analysis of the components of the Nasonov gland in *Apis mellifera* (3 colonies, each colony tested 7 bees) and *Apis cerana* (3 colonies, each colony tested 7 bees). The table show the mean ± standard error. Note: significance level (**$p < 0.01$, *$0.01 < p < 0.05$, ns $p > 0.05$).

Peaks	Structure	Quantities of *Ac* (ng/insect)	Quantities of *Am* (ng/insect)	Sig
1	(E)- Citral	0.00	2.536 ± 1.849	**
2	(*Z*)- Citral	0.00	4.737 ± 3.813	**
3	Nerol	0.00	1.742 ± 0.699	**
4	Geraniol	0.00	254.786 ± 263.219	**
5	Geranic Acid	0.00	197.041 ± 119.634	**
6	Nerolic Acid	0.00	11.790 ± 12.001	**
7	(*E,E*)- Farnesol	0.00	255.129 ± 131.738	**
8	n-Octanoic Acid	6.804 ± 6.482	0.983 ± 0.793	**
9	Decanoic Acid	4.714 ± 3.316	2.838 ± 2.184	ns
10	Dodecanoic Acid	5.443 ± 2.774	3.518 ± 1.355	*
11	Tetradecanoic Acid	4.126 ± 2.593	4.638 ± 2.156	ns
12	Hexadecanoic Acid	13.415 ± 5.340	64.715 ± 24.048	**
13	*cis*-9-Hexadecanoic Acid	3.115 ± 1.751	16.106 ± 10.152	**
14	Octadecanoic Acid	9.665 ± 4.556	9.877 ± 4.580	ns
15	Oleic Acid	6.205 ± 4.748	71.642 ± 24.823	**
16	Linoleic Acid	19.922 ± 14.062	17.720 ± 10.964	ns
17	Linolenic Acid	12.039 ± 6.288	12.655 ± 7.922	ns

2.3.1. Statistical analysis

We used Chemstation software (Agilent Technologies, USA) to collect and analyze chemical data. All compounds were identified by comparing their retention times to those of the standards. The differences of compound quantities in the Nasonov glands of *A. cerana* and *A. mellifera* were compared using a *t*-test. For analysis of the chemical profile of worker bee bodies at different time points after death, we used a Univariate Repeated-Measures Analysis of Variance (ANOVA) with bees as the repeated measure. We also used ANOVA to determine the effect of these treatment on the removal of the corpse and aggressive behaviors and used Tukey’s Honestly Significant Difference (HSD) test for post-hoc comparisons.

3. Results

3.1. Chemical analysis of the A. cerana Nasonov gland

3.1.1. Pheromone chemical identification

The *A. cerana* gland contained all 10 fatty acids although in different proportions from *A. mellifera*.

In total, 17 compounds were found between the Nasonov glands of *A. mellifera* and *A. cerana*. 10 of the compounds were fatty acids and 7 of

the compounds were terpenoids found only in the *A. mellifera* gland. Because some organic acids were not suitably separated by the HP-5 capillary columns, an HP-FFAP capillary column was used. Each chemical was identified by using standards. After optimization of the conditions, the fatty acids with longer carbon chains were separated by extending the retention time. The acids have been identified as organic acids: n-octanoic acid ($C_{8:0}$), decanoic acid ($C_{10:0}$), dodecanoic acid ($C_{12:0}$), myristic acid (tetradecanoic acid, $C_{14:0}$), palmitic acid (hexadecanoic acid, $C_{16:0}$), Cis-9-hexadecenoic acid $\Delta^9C_{16:1}$), stearic acid (octadecanoic acid ($C_{18:0}$), oleic acid ($\Delta^9C_{18:1}$), linoleic acid ($\Delta^{9,12}C_{18:2}$), and linolenic acid ($\Delta^{9,12,15}C_{18:3}$). No terpenoids were detected in the extracts of the Nasonov gland of *A. cerana*.

3.1.2. Pheromone quantification analysis

A comparison of the quantification of components in the Nasonov glands of *A. mellifera* and *A cerana* was done. *A. mellifera* contains seven terpenoids, their ratios in the gland were detailed in previous works (1: 1: 1: 100: 75: 12: 50, Free et al., 1981). These terpenoids were not detected in the Nasonov gland extracts of *A. cerana*. However, the same fatty acids were all found in the Nasonov gland extracts of *A. mellifera* and *A. cerana*. Nearly all of the acids are found in higher amounts in the Nasonov gland of *A. mellifera*. The only exception was n-octanoic acid. This acid was found in a higher quantity in *A. cerana*.

3.2. Analysis of the chemical profile of worker bee bodies at different time points after death

The fatty acid composition of *A. cerana* at different points of time after death are as follows:

As shown in Fig. 2, fatty acids were found, via SPME, to change in amount in *A. cerana* after death. While the changes in fatty acid levels were modest for the first 32 h after death, the release of fatty acids in *A. cerana* did increase after death. Most chemicals were rising up to around 64 h after death. The amount of 4 components (Tetradecanoic acid: $F_{9,36} = 3.02$, $P < 0.05$, Hexadecanoic acid: $F_{9,36} = 3.25$, $P < 0.05$, *cis*-9-Hexadecenoic acid: $F_{9,36} = 3.34$, $P < 0.05$, Octadecanoic acid: $F_{9,36} = 2.98$, $P < 0.05$) significantly increased the earliest, peaking at 128 h, and then a sharp decline followed. For other components, only, the amount of n-Octanoic acid and Linoleic acid showed a significant difference at 256 h, Decanoic acid and Linolenic acid showed no significant difference in these time points after death. These results show that fatty acids increase in quantity in *A. cerana* after their death. They also show that the particular chemical blend of acids present in a bee during life, begins shifting immediately after death.

3.3. Behavioral bioassay

To quantify the removal response when *A. cerana* bees were rinsed in dichloromethane, a bee was presented with two concentrations of oleic acid or a synthetic blend of *A. cerana* Nasonov pheromone. Different treatments elicited significantly different responses ($F_{4,580} = 63.59$, $P < 0.05$, the Nasonov gland dummies were removed significantly more often than the oleic acid treated dummies, Fig. 3).

Aggression is another response to unsaturated fatty acids, thought to be associated with predator avoidance. There is a crushed conspecific body, body part, or hemolymph this can cause aggressive behaviors in some insects, potentially a cue that there is a predation threat (Yao et al., 2009). We considered aggressive behavior to include bees antennating the corpse then spinning around and fanning, bees charging the corpse, grasping the corpse and dragging it to a new spot on the landing board, and bees moving the corpse out of the test area. Different treatments elicited significantly different responses ($F_{4,2252} = 61.48$, $P < 0.05$, the Nasonov gland dummies were caused significantly more aggressive than the oleic acid treated dummies) as shown in Fig. 4.

4. Discussion

4.1. Chemical analysis of the A. cerana Nasonov gland

In contrast to Abdullah et al. (1990) and Naik et al. (1988), our analysis of the Nasonov gland of *A. cerana* showed none of the terpenoids found in the *A. mellifera* gland (Fig. 1). It's been widely thought that the terpenoids were largely responsible for the attractiveness of the Nasonov gland and critical to its role as a swarm attractant guiding pheromone (Schmidt et al., 1993). Nasonov pheromone is also thought to play an important role in the clustering process in swarming (Free et al., 1981). Our results raise the question as to if the function of the Nasonov gland in *A. cerana* is the same as that of *A. mellifera*. These initial results caused us to evaluate possible alternative functions for a pheromone comprised of fatty acids. A possibility is that the Nasonov gland in *A. cerana* functions as a source of necromone. These fatty acids are found in the necromones of different insect and crustacean phyla (Yao et al., 2009).

4.2. Analysis of the chemical profile of worker bee bodies at different time points after death

We found that the levels of fatty acids contained in the Nasonov gland of *A. cerana* all began changing in the worker bee bodies after

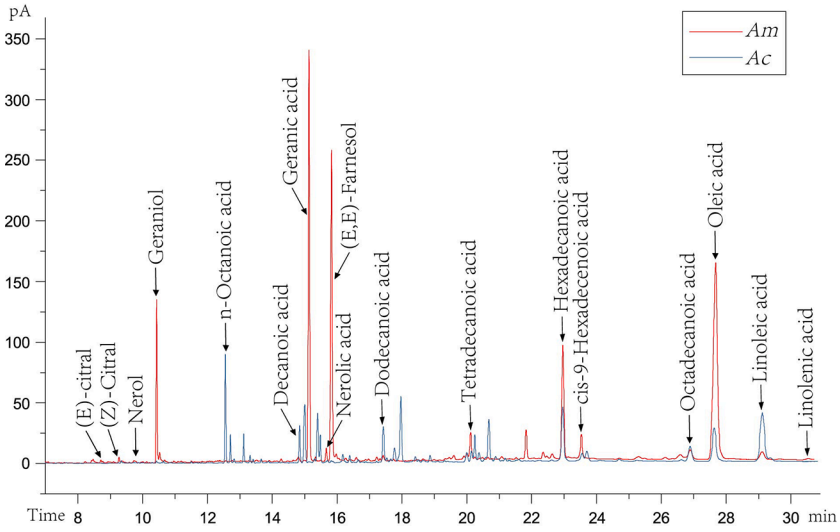

Fig. 1. Comparison of the GC profile of 1 *Apis mellifera* and 1 *Apis cerana* Nasonov gland compounds. In total there were 17 compounds found using an HP-FFAP column. In *A. mellifera*, there were 7 terpenoids found: (*E*)-Citral, (*Z*)-Citral, Nerol, Geraniol, Geranic acid, Nerolic acid, and (*E,E*)- Farnesol. There were also 10 fatty acids found: n-Octanoic acid, Decanoic acid, Dodecanoic acid, Tetradecanoic acid, Hexadecanoic acid, *cis*-9-Hexadecanoic acid, Octadecanoic acid, Oleic acid, Linoleic acid, and Linolenic acid. In *A. cerana*, only the 10 fatty acids were found.

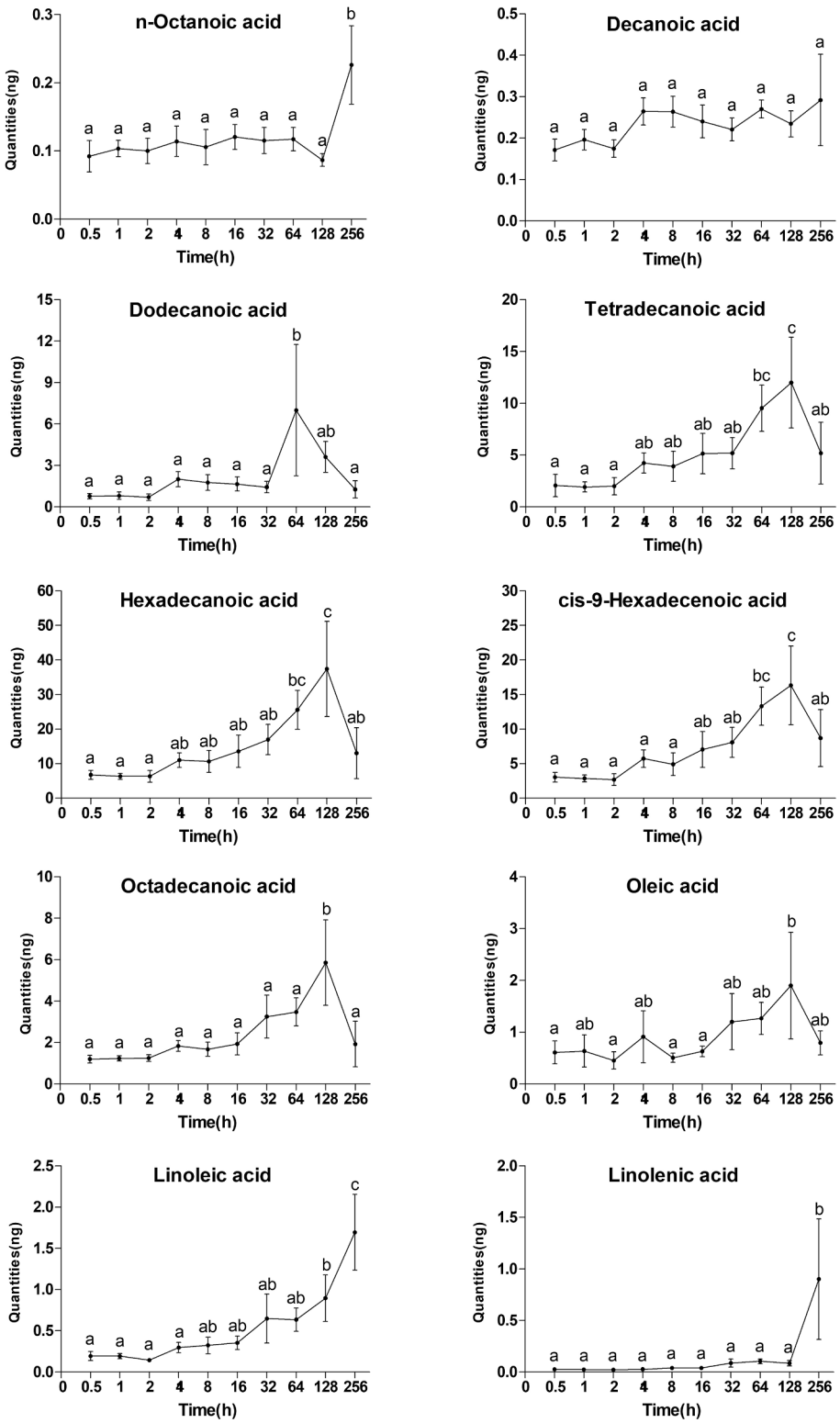

Fig. 2. The change in fatty acid quantities after death in *A. cerana* (five bees from five colonies were tested). Graphs show the mean ± standard error. Different letters indicate significant differences (Tukey's HSD test, $P < 0.05$).

death. The Nasonov gland could be releasing these acids via the microtubules that produce the glandular secretions. They might lose their ability to contract and regulate, and their porous structure opens up. Even during the life of the bee, the Nasonov gland lacks a definite closing mechanism (Jacobs, 1925; Renner, 1960). Fatty acids are non-volatile and they are emitted from cells or corpses by bacterial or enzymatic processes (Yao et al., 2009). Although the amount of each fatty acid released after death were different, the increasing and decreasing trends were similar across all acid types.

It is possible that *A. cerana* worker bees sense the body of the dead bees through the odor of the fatty acids being released after death. It should be noted that in our experiment and the work of others, the

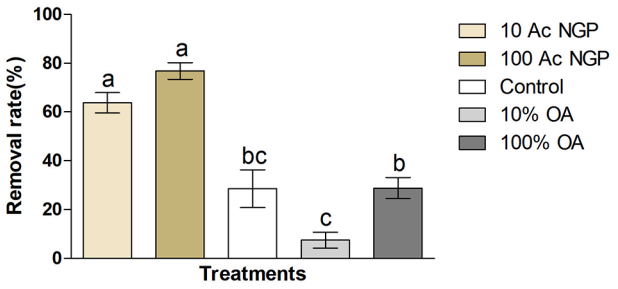

Fig. 3. The rate of removal of the two different chemicals, oleic acid, a synthetic blend of *A. cerana* Nasonov pheromone (Ac NGP) and a control. Graphs show the mean ± 1 standard error. Different letters indicate significant differences (Tukey's HSD test, $P < 0.05$).

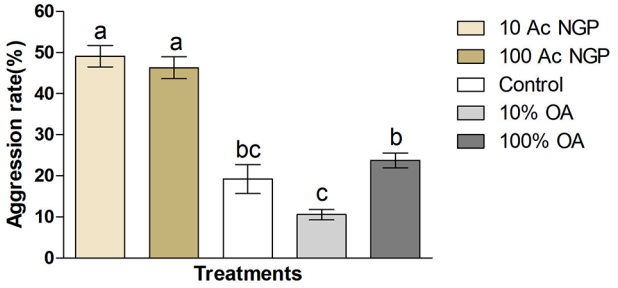

Fig. 4. Effect of different treatments on scentless corpses that stimulated aggressive behavior by bees at their entrance. Mean ± 1 standard error are shown. Different letters indicate significant differences (Tukey HSD test, $P < 0.05$).

removal of honeybee corpses occurs long before the fatty acids begin to reach their peak levels after death (Visscher, 1983). However, peak levels are likely not required to begin undertaking. Even small shifts in fatty acid combinations may contribute to a change in the recognition template reflecting colony membership (Buchwald and Breed, 2005). This would alert the undertaker bees that there was a threat from the deceased conspecific, which would cause them to remove the corpse.

The brains of guards and undertakers are nearly the same and changes in acid levels that affect guards would likely have an effect on undertakers (Cash et al., 2005). Furthermore, it has been shown that hygienic honeybees have "an enhanced ability to detect oleic, linoleic, and the oleic acid ester" (Martin et al., 2002). This shows that hygienic bees should be better equipped physiologically to engage in undertaking behavior and remove those bees which are chemically unbalanced. *A. cerana* is well known for its high levels of hygienic behavior (Lin et al., 2016). It has been shown in *A. mellifera* that there is significant overlap between hygienic specialists and undertaker bees (Perez and Johnson, 2019). Perez and Johnson posed the question of whether the same death cue was involved in undertaking and hygienic behavior or whether the superior sense of smell in hygienic bees could drive high detection of different death cues in either task. While more work is needed to confirm, our preliminary results seem to support their second hypothesis.

4.3. Behavioral bioassay

Our work suggests that oleic acid may not be the "death cue" for *A. cerana*, as it is commonly referred to in certain other bee species and other Hymenoptera (Wilson et al., 1958; Yao et al., 2009; Sun and Zhou, 2013; Mcafee et al., 2018). Oleic acid is widely regarded as the only known shared death chemical between multiple species (Sun and Zhou, 2013). Yet, at both high and low concentrations, oleic acid by itself did not stimulate removal. The fact that *A. cerana* bees did not remove the oleic acid treated dummies but did remove the dummies with the Nasonov blend, shows that they possess a sensitivity to these fatty acid blends, but that oleic acid on its own is not a primary cue that elicits necrophoric behavior. Interestingly, oleic acid is abundant on newly emerged bees that have not yet been widely exposed to CHCs through colony wax (Breed, 1998). Thus, after washing the other CHCs off of dummy bees and only applying oleic acid, this might explain why oleic acid did not trigger a response in guard or undertaking bees.

Another study also found the African termite *Pseudacanthotermes spiniger*, would not engage in undertaking burial behavior when exposed to oleic acid alone. Their study showed a complex mixture of fatty acids, indol, and phenol together stimulated burial behavior in the termites. It was only through mixtures of chemicals that the termites reacted (Chouvenc et al., 2011). In another study with ants, there were repellent effects from oleic and linoleic acid, but no effects were seen from lauric, myristic, palmitic, and stearic acids (Dani et al., 1996). It's possible that oleic acid is part of a blend of fatty acids that successfully stimulate necrophoresis in *A. cerana*, but that alone the compound is lacking the synergistic cue necessary to instigate the behavior. Furthermore, it has been observed that the latent period between death and removal is very short for only fatty acid death cues to be responsible (Howard and Tschinkel, 1976). There is potentially more cuticular chemistry involved in the cue and this needs to be investigated further.

The *A. cerana* Nasonov blend however, had a significant effect on the removal of dummies. This indicates that the blend of chemicals inside likely play a role in stimulating the removal of corpses from a hive. However, because there was not 100% removal of the corpses treated with the synthetic Nasonov fatty acid blend, it is possible that there is an outside influence from other unknown chemicals as well.

When dividing the behaviors into two groups, of either non-aggressive or aggressive, we found that the Nasonov blend caused the bees to act aggressively nearly half of the times that they interacted with the corpses. This was different from pure oleic acid, which rarely caused an aggressive reaction. Maybe the Nasonov pheromone in *A. cerana* no longer functions as a guiding pheromone but as a defense signal to guards as in *Apis dorsata* (Kastberger et al., 1998). Or maybe the chemicals found in the gland cause specific behaviors that are contextually dependent, as Gordon found in her experiments with ants and oleic acid (Gordon, 1983). Or potentially, the composition of the signal itself has changed after death. In social insects, there can be signals that are produced prior to death that change in chemical quantity after death and thus change in function as well (Sun et al., 2018).

To our knowledge, this is the first report in *A. cerana* linking the Nasonov gland synthetic blend to a social immunity function. The limitation of this bioassay was that we could not test every fatty acid individually in this way. We don't yet know the extent of the connection between the release of the fatty acids from the nasonov gland on the corpse of *A. cerana* stimulating undertaking behavior and the other aggressive behaviors recorded. Next step for future research is to design a bioassay to continue testing the other individual fatty acids present in the *A. cerana* Nasonov glands and incorporate some cuticular hydrocarbons into the testing of fatty acid blends. Recently, a study reported the differences in chemical cuticles between dead and living *A. cerana* workers, the results showed the emission of cuticular hydrocarbons was reduced in dead *A. cerana* bees, this change is caused by the cooling of dead bees (Wen, 2020). This change in levels of CHCs may also contribute to the changing recognition template along with the changing levels in fatty acids after death. Our goal in this study was to identify the components of the Nasonov gland and explore their functions in *A. cerana*, while our evidence indicates that the fatty acids found in the gland do play a role in undertaking, we don't exclude the possibility of other death cues such as cuticular profiles and this should be explored in future research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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