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RAPID COMMUNICATION

Alarm Pheromone Induces Immediate–Early Gene Expression and Slow Behavioral Response in Honey Bees

Cédric Alaux · Gene E. Robinson

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Abstract Primer and releaser pheromones are molecules used for communication that induce species-specific responses. In contrast to primer pheromones, it is not known whether the quicker-acting releaser pheromones can affect brain gene expression. We show here that isopentyl acetate (IPA), a releaser pheromone that communicates alarm in honey bees, not only provokes a quick defensive response but also influences behavior for a longer period of time and affects brain gene expression. Exposure to IPA affected behavioral responsiveness to subsequent exposures to IPA and induced the expression of the immediate early gene and transcription factor *c-Jun* in the antennal lobes. Our findings blur the long-standing distinction between primer and releaser pheromone and highlight the pervasiveness of environmental regulation of brain gene expression.

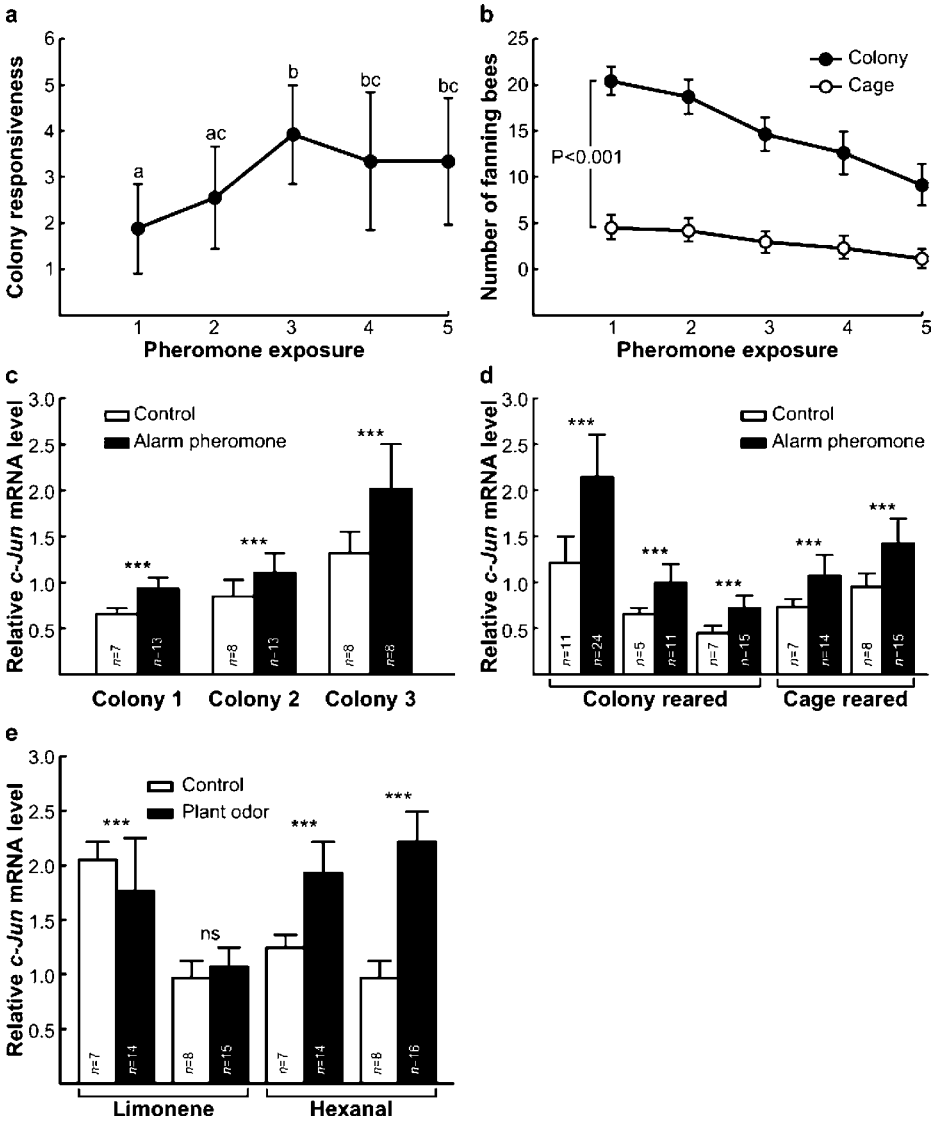
Keywords Pheromones · Immediate early gene · Antennal lobes · Behavioral plasticity · Honey bee · *c-Jun*

Introduction

Releaser pheromones cause immediate and short-term responses in organisms, whereas primer pheromones cause physiological changes that later lead to behavioral modifications. Primer pheromones exert their effects, in part, by causing changes in brain gene expression (Grozinger et al. 2003). However, it is not known whether the effects of releaser pheromones are too short-lived to work via changes in gene expression. With growing evidence for social regulation of brain gene expression in a variety of contexts (Robinson et al. 2005), we decided to test whether a releaser pheromone could affect gene expression.

C. Alaux (✉) · G. E. Robinson
Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Drive, Urbana, IL 61801, USA
e-mail: calaux@life.uiuc.edu

G. E. Robinson
Department of Entomology, Neuroscience Program and Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA



We used isopentyl acetate (IPA), the principal component of the sting alarm pheromone in the honey bee, *Apis mellifera*, which elicits a response similar to that of the whole pheromone blend (Boch et al. 1962). Alarm pheromone provokes rapid defensive behavior, although longer-term effects are suggested by the observation that colonies that have been disturbed apparently remain aroused over a longer period (Winston 1987). Colony defense in honey bees typically begins with the release of alarm pheromone by "guard bees" at the hive entrance, which then provokes other bees to move to the entrance to face the intruder(s).

We first tested the effect of alarm pheromone exposure on longer latency behavior by repeatedly exposing bees to IPA in the field or laboratory. We then measured the effect of IPA exposure on the expression of the immediate early gene (IEG) and transcription factor, *c-Jun*, in the antennal lobes (ALs), the primary olfactory centers in the insect brain. Immediate early genes are involved in synaptic plasticity and are known to link experience

Fig. 1 Effect of exposure to alarm pheromone, or other odors, on honey bee behavior and *c-Jun* brain expression. **a** Repeated exposure to IPA caused a significant increase in colony arousal under natural conditions (two-way repeated measures ANOVA, colony: $F_{6,17}=81.92$, $P<0.001$, time exposure: $F_{4,68}=6.87$, $P<0.001$, interaction colony × time exposure: $F_{24,68}=0.88$, $P=0.621$). *Different letters* indicate significant differences in colony responsiveness over time (Tukey *post hoc* test, 1 vs. 3: $P<0.001$, 2 vs. 3: $P<0.01$, 1 vs. 4 and 5: $P<0.05$). Similar results were obtained for analyses based on number of bees responding relative to the first response (colony: $F_{6,17}=1.18$, $P=0.363$, time exposure: $F_{4,68}=3.09$, $P<0.001$, interaction colony × time exposure: $F_{24,68}=1.24$, $P=0.239$). The number of bees at the nest entrance ranged from 6 to 58 bees, and the mean lowest and highest responses were 30.7±13.5 and 65.4±23 bees, respectively. **b** Repeated exposure to IPA caused a significant decrease in responsiveness in the laboratory (rearing condition: $F_{1,18}=512.03$, $P<0.001$; time exposure: $F_{4,72}=94.31$, $P<0.001$; rearing condition × time exposure: $F_{4,72}=26.15$, $P<0.001$). Assays performed on caged bees reared either in typical colonies in the field or in laboratory cages (both $N=10$ cages). Isopentyl acetate-induced increase in *c-Jun* expression in the ALs of bees in typical colonies in the field (**c**) and laboratory cages (**d**). **e** Effect of exposure to known plant odors (limonene or hexanal) on *c-Jun* expression. Tests were performed as for IPA in the laboratory; colony-reared bees were exposed for 60 sec to 5 μl of neat odorant. We performed two trials, each with one control cage (filter paper alone) and two treatment cages, for each odor ($N=7$–8 individuals analyzed/cage). *Triple asterisks* represent $P<0.001$, Mann–Whitney rank sum test. Data show mean±SD. *Numbers in bars* show numbers of individual brains assayed

to changes in gene expression (Clayton 2000). The IEG *c-Jun* is expressed in neural circuits involved in olfaction in mammals (Baba et al. 1997).

Methods and Materials

Effect of Alarm Pheromone on Behavior Bees were exposed to a 60-sec presentation of 2.5 μl IPA, diluted in mineral oil (1/10) (Sigma-Aldrich, St Louis, MO, USA) (Collins and Rothenbuhler 1978), every 30 min ($N=5$ successive exposures). Mineral oil alone did not elicit a response (data not shown).

In the field assay, the pheromone solution was applied on a filter paper presented at the hive entrance ($N=7$ colonies, 2–4 replicates/colony, 24 replicates total). To measure colony arousal, the number of responding bees was determined from photographic snapshots of the hive entrance 0, 15, 30, 60, and 120 sec before and 15, 30, 60, and 120 sec after IPA exposure. Colony arousal was measured as the difference between the mean number of bees at the hive entrance before and after IPA exposure. The intensity of colony response at each exposure was ranked from lowest (rank 1) to highest (rank 5).

The laboratory assay was performed with cages of 25 8-d-old bees reared either in a typical colony in the field or in cages in the laboratory. Cage-reared bees were removed from their hive prior to eclosion by transferring honeycomb frames containing pupae to an incubator (33°C). They were then placed in cages upon eclosion and supplied with honey, pollen, and water. The cages were kept under a cardboard box (with holes on each side) at 32±2°C, 70–90% relative humidity (RH), and 12:12-hr light/dark photoperiod. Both colony- and cage-reared bees were transferred to test cages 4 hr prior to testing. This was done to minimize the effects of any exposure to alarm pheromone that might have occurred during transfer (IEG expression returns to basal levels 4 hr after stimulation; Clayton 2000). The IPA-treated filter paper was inserted under the wire floor of a Plexiglas cage (10×10×7 cm), and the wing fanning response was quantified. This assay has been shown to give responses that correlate with field behavior (Collins and Rothenbuhler 1978). Tests were conducted in a light room at 28±2°C and 30–40% RH.

Effect of Alarm Pheromone on IEG Expression *c-Jun* expression was analyzed 30 min after one exposure to IPA, a timescale appropriate for detecting an increase in IEG mRNA

(Clayton 2000). In the field experiment, bees standing at the hive entrance were randomly collected before IPA exposure (control); then, the colony was exposed to IPA for 60 sec, and the bees that rapidly approached the filter paper and behaved aggressively toward other bees were collected and placed in a Plexiglas cage for 30 min. In the laboratory experiment, bees exposed to either mineral oil (control) or IPA were frozen in liquid nitrogen 30 min after exposure. We performed three trials of the field assay, three trials of the laboratory assay with colony-reared bees (each one with one control cage and two treatment cages, N=5–12 individuals analyzed/cage), and two trials of the laboratory assay with cage-reared bees (each one with one control cage and two treatment cages, N=7–8 individuals analyzed/cage). Bees were held at −80°C until AL dissection and mRNA quantification by real-time quantitative reverse-transcriptase PCR (Grozinger et al. 2003). *c-Jun* mRNA levels were normalized to an mRNA loading control "housekeeping" gene (*rp49*). Primer sequences (5′ to 3′) were *c-Jun*, forward: CGTGGCGGCATCCAAA; *c-Jun*, reverse: CCCTTCAGCAATTTAACCTTATCTTC; (control gene) *rp49*, forward: GGAACT GGAAGTTTTAATGATGCA; and *rp49*, reverse: CAACAATGGATTTACGTTTT TTACTG. *c-Jun* expression was also determined following exposure to the known plant odorants, limonene and hexanal (Sigma-Aldrich).

Results and Discussion

Isopentyl acetate exposure affected subsequent behavioral responses of bees to alarm pheromone. There was a significant increase in the number of bees at the hive entrance over the first 1.5 hr before the response leveled off (Fig. 1a). Isopentyl acetate exposure also affected behavior in the laboratory but opposite to the field experiment; both colony- and cage-reared bees showed a significant decrease in responsiveness for at least 2.5 hr in response to IPA (Fig. 1b). The laboratory response of cage-reared bees was greatly attenuated relative to colony-reared bees (Fig. 1b). This is consistent with previous findings on the effects of queenlessness and social isolation on brain development (Gascuel and Masson 1987; Morgan et al. 1998).

The exposure of typical honey bee colonies in the field to IPA caused a significant increase in *c-Jun* expression (Fig. 1c). An equivalent *c-Jun* induction was also seen in response to IPA exposure in the laboratory, despite the opposite behavioral responses observed in the field and laboratory (Fig. 1a, b). The *c-Jun* response occurred equivalently in both colony- and cage-reared bees (Fig. 1d), although the behavioral response was stronger in colony-reared bees.

We tested the selectivity of this IEG response by exposing bees in cages to two other biologically relevant volatile odors: hexanal, a ubiquitous component of green leaf volatiles, and limonene, a floral odor. Hexanal induced *c-Jun*, but limonene did not (Fig. 1e). Speculation on the reasons for these contrasting results is beyond the scope of the present study; nevertheless, our results indicate that this particular IEG response in bees is not specific to alarm pheromone but is selective.

This is the first demonstration that a releaser pheromone can affect longer-latency behavioral response probabilities and cause changes in brain gene expression. These results demonstrate a genomic response to a releaser pheromone that is behaviorally related, although the genomic response appears to be less sensitive to behavioral context than does the behavior itself. *c-Jun* induction may initiate the response to alarm pheromone in ALs,

but integration with other mechanosensory and visual stimuli relevant to colony defense, present in the field but absent in the lab environment, apparently occurs at higher levels of the brain to shape the behavioral response (Li and Strausfeld 1999). Our findings blur the long-standing distinction between primer and releaser pheromones and highlight the pervasiveness of social regulation of brain gene expression (Robinson et al. 2005).

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