

Pilocarpine improves recognition of nestmates in young honey bees

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ABSTRACT

Honey bees can distinguish nestmates from non-nestmates, directing aggressive responses toward non-nestmates and rarely attacking nestmates. Here we provide evidence that treatment with pilocarpine, a muscarinic agonist, significantly reduced the number of aggressive responses directed toward nestmates. By contrast, treatment with scopolamine, a muscarinic antagonist, significantly increased attacks on nestmates. Locomotor activity was not altered by these pharmacological treatments. When interpreted in light of known cholinergic pathways in the insect brain, our results provide the first evidence that cholinergic signaling via muscarinic receptors plays a role in olfaction-based social behavior in honey bees.

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Honey bee workers use odor cues to distinguish nestmates from non-nestmates [6]. These cues are based on heritable (genetically determined composition of cuticular hydrocarbons and comb wax) and nonheritable environmental odors (floral oils, pollen) present in each hive in a unique combination [5,10,22]. All colony members share the same odor cue profile [9]. Guard bees positioned at the nest (hive) entrance accept or attack incoming bees based on this profile [4]. Bees attacked as foreign are pushed, bitten, and sometimes stung. Bees that are accepted pass unmolested into the hive [3]. Guarding at the hive entrance is a specialized task performed by a small fraction of workers in a colony [22], but numerous studies have shown that adult bees in general are significantly more likely to attack a non-nestmate than a nestmate [7].

In many mammals, such as voles [39] and sheep [25], kin recognition is based on learned odor cues. Pharmacological studies have shown that signaling via muscarinic receptors is required for odor-based kin recognition in some species [30]. Ewes, for example, form an exclusive olfactory bond with their lambs and do not allow unfamiliar lambs to suckle. Activity in cholinergic pathways is critical to the formation of this olfactory memory, as injection of ewes with the muscarinic antagonist scopolamine at the time of parturition significantly increased the proportion of ewes that accepted an

alien lamb in a later selectivity test [26]. More recently, numerous studies in mammals have demonstrated that disruption of muscarinic signaling results in the disruption of many forms of odor memory [40].

Acetylcholine (ACh) also plays an important role in behavior, memory and neural plasticity in bees [16,23,27,28,35]. In the insect brain, ACh is a major neurotransmitter in sensory neurons and many interneuron populations [1,24]. In particular, information from the antennal lobes (primary olfactory neuropil) is transmitted to the mushroom bodies, a higher order brain region involved in multi-modal sensory integration and certain forms of learning and memory, by a cholinergic projection [11]. Intracranial injections of scopolamine 20 min after one trial classical conditioning of the proboscis extension reflex (CS, vanillin; US, drop of sugar water) significantly reduced the percentage of bees responding to the CS from 5 to 20 min after the injection, although responding returned to control levels 1 h post-treatment [16]. Injections of scopolamine into the vertical (alpha) lobes of the mushroom bodies were particularly effective in disrupting retrieval of a learned association of an odor with a food reward [27].

In the present study, we used a well-established laboratory assay of nestmate recognition to assess possible cholinergic mediation of odor-based nestmate recognition in the bee [4,5,7,8]. If signaling via muscarinic receptors is important for acquisition and/or retrieval of an odor template associated with nestmates, blockade of central muscarinic receptors with scopolamine is predicted to impair recognition of nestmates. Conversely, stimulation of brain muscarinic receptors with pilocarpine, a muscarinic agonist, is

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predicted to improve the ability to discriminate nestmates from non-nestmates.

Honey bees for these studies were obtained from research apiaries maintained at the University of Illinois at Urbana Champaign (Urbana, IL, USA) and Wake Forest University (Winston-Salem, NC, USA). Behavioral tests were performed during the summers of 2004, 2005, and 2006. Experimental bees were derived from unrelated colonies containing a mixture of European races. Worker bees were obtained by removing honeycomb-filled brood frames containing pupae from source colonies in the field and placing them in an incubator (33–34 °C, 85–95% RH). Bees that completed metamorphosis and emerged within a 24 h period from a single colony were introduced in groups of 10–12 nestmates to plastic Petri dishes (90 mm diameter, 10 mm deep, with an 8 mm hole drilled in the lid for feeding tube access). We chose to work with young bees because, in preliminary studies using our test conditions, older bees ($N=188$) with foraging experience almost never attacked nestmates, making it impossible to detect a treatment-related improvement in performance.

Each dish contained a small piece of comb (25 mm²) from their natal colony. Previous studies have shown that even 1 h of contact with brood comb permits newly emerged bees to learn the unique odor of their colony [8]. These dishes were stored in darkness (mimicking conditions within the hive) in the incubator for the next 5 days. Nestmates were defined as all bees that emerged from a brood frame derived from a single source on the same day; non-nestmates were bees that emerged on the same day from a brood frame obtained from a different source colony. To prevent cross-contamination of wax-based cues, all equipment and supplies that contacted comb were kept separate throughout the entire testing procedure.

Bees designated to serve as responders in nestmate recognition assays were treated orally with a 1:1 solution of sucrose and water (control), pilocarpine dissolved in sucrose (muscarinic agonist; Sigma P6503, 10⁻⁴ M), or scopolamine dissolved in sucrose (muscarinic antagonist; Sigma S1013, 10⁻³ M) for 5 days; bees designated to serve as stimulus bees were marked with a dot of paint (Testor's PLA) on the dorsal thorax at the time the dishes were established and fed 50% (w/v) sucrose. Doses for the cholinergic drugs were 10-fold lower than the LD₅₀ for each drug (data not shown). Pilocarpine and scopolamine have previously been shown to be active at the insect muscarinic receptor [15,23,38]; it should be noted that the honey bee genome encodes a single G-protein-coupled muscarinic-type cholinergic

receptor, rather than the multiple subtypes characteristic of vertebrates [20,21]. Feeding tubes (inverted 1.5 ml polypropylene microcentrifuge tubes with holes punched in the tip with an 18 gauge needle) were changed daily; fresh solutions were made weekly.

The assay used in this study assesses the response of a group of bees to a single introduced nestmate or non-nestmate. On the 5th day after the dishes were established, a single sucrose-fed nestmate or non-nestmate was added to each dish. The number of aggressive interactions was recorded over a 4-min period in tests conducted in 2004, and over a 3-min period in tests conducted in 2005/2006. Observations began immediately after the introduction. Behavioral observations were made under dim red illumination, to which bees are blind because they lack a red-sensitive photopigment [29]. A total of 722 (260 control, 243 pilocarpine and 219 scopolamine) dishes were tested. For statistical analysis, each dish was treated as a single data point; any observed grappling, biting, or stinging resulted in the dish being categorically scored as rejecting the introduced bee; dishes with no observed aggressive responses during the observation period were scored as accepting the introduced bee. Our expectation was that nestmates would be accepted and non-nestmates would be rejected. Most trials, even those scored as rejecting because of observed aggression, ended with acceptance of the introduced bee, but in some cases the introduced individual was killed. The G^2 statistic was calculated for the resulting 2 × 2 frequency tables using StatView 4.5.1 for Macintosh (Abacus Concepts, Inc.).

Locomotor responses were recorded in bees reared under similar conditions and treatment schedules. Locomotor activity was defined as the number of times any bee within the dish crossed a center line over a 4-min period.

Control bees responded differently to nestmates than to non-nestmates ($G^2=21.6$, d.f. = 1; $p<0.0001$), as shown in previous studies [2]. Pilocarpine-treated bees also responded differently to nestmates and non-nestmates ($G^2=63.9$, d.f. = 1; $p<0.0001$), as did scopolamine-treated bees ($G^2=8.4$, d.f. = 1; $p<0.01$).

Further analyses, however, revealed important differences among the three test groups (Fig. 1). Pilocarpine-treated bees were significantly less likely to react aggressively toward a nestmate than control bees ($G^2=4.4$, d.f. = 1, $p<0.05$). Pilocarpine-treated bees also made fewer total errors (rejection of nestmate + acceptance of non-nestmate) than did controls ($G^2=7.4$, d.f. = 1; $p<0.01$) (Fig. 2). They did not differ from controls on the overall proportion of trials featuring displays of aggression.

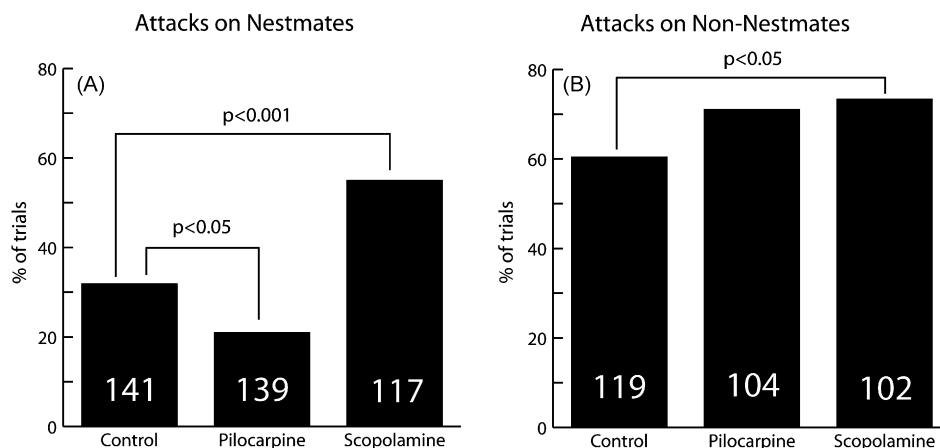


Fig. 1. Percentage of trials resulting in attacks on an unfamiliar stimulus bee. The G^2 statistic was used to analyze contingency tables. Comparisons yielding a statistically significant difference and associated p -values are indicated by brackets. Numbers in bars indicate the number of dishes tested. (A) Attacks on nestmates. (B) Attacks on non-nestmates.

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