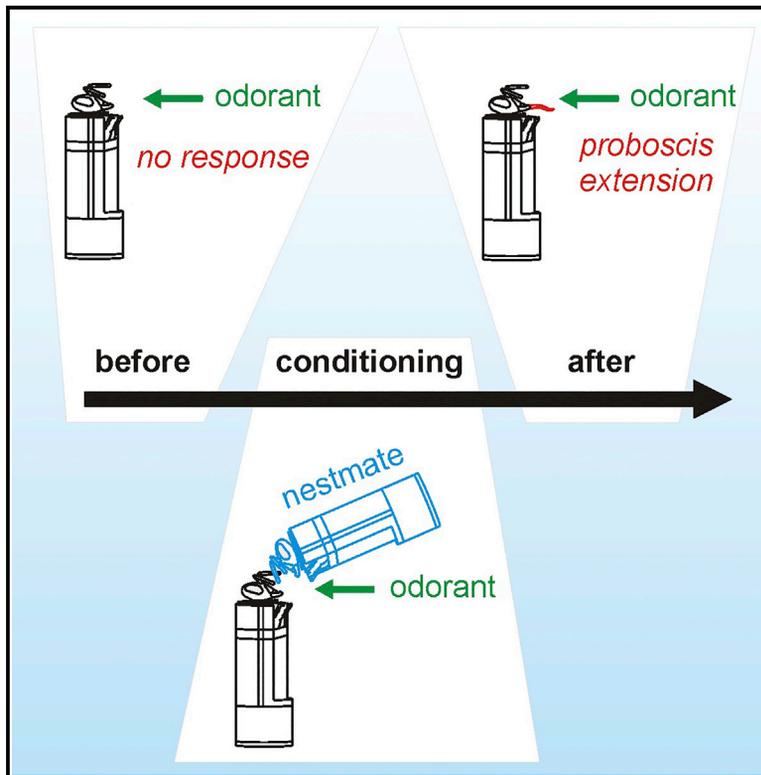# Current Biology

Social Contact Acts as Appetitive Reinforcement and Supports Associative Learning in Honeybees

Graphical Abstract

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In Brief

Using behavioral experiments and antenna movement recordings, Cholé et al. uncover a novel social learning process in honeybees. Bees learn to associate originally neutral stimuli (odorants) with contact with a nestmate, which acts as an appetitive reinforcement. This reinforcement is mediated by bees' antennal communication.

Highlights

- Contact with a nestmate elicits an appetitive response (PER) in restrained bees

- Contact with a nestmate acts as a reinforcement in odor-nestmate PER conditioning

- Social reinforcement is mediated by bees' antennal communication

- This form of social learning may facilitate resource exploitation by social insects

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Social Contact Acts as Appetitive Reinforcement and Supports Associative Learning in Honeybees

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SUMMARY

Social learning is taxonomically widespread in the animal kingdom [1], and although it is long thought to be a hallmark of vertebrates, recent studies revealed that it also exists in insects [2–5]. The adaptive functions of social learning are well known, but its underlying mechanisms remain debated [2, 5, 6]. Social insects critically depend on the social transmission of information for successful food search and their colonies' fitness [7] and are tractable models for studying the social cues and cognitive mechanisms involved [2–5]. Besides the well-known dance language allowing them to communicate the location of food sources among nestmates [8], honeybees also learn chemosensory information about these sources both outside and within the hive [9, 10]. In the latter case, they associate the floral scent carried by returning foragers on their body with the nectar provided through mouth-to-mouth trophallaxis, similar to the manner in which foragers directly learn odorant-nectar reward associations at the foraging patch [9–11]. Strikingly, however, neither the dance nor trophallaxis is strictly necessary for foragers recruited within the hive to find the right floral source, and simple body contact between foragers may be sufficient [12]. What is the reinforcing agent in this case? We show here that simple social contact acts as appetitive reinforcement and can be used in associative olfactory learning. We demonstrate that this social reinforcement is mediated by bees' antennal movements and modulated by bees' behavioral development. These results unveil a social learning mechanism that may play a facilitating role in resource exploitation by social groups.

RESULTS AND DISCUSSION

Restrained Honeybees Show an Appetitive Response to Contact with a Nestmate

For the last fifty years, honeybees have represented a central model for the study of appetitive learning and memory, through the well-known Pavlovian conditioning of the proboscis extension response (PER) [13]. When the antennae of a restrained, hungry bee come into contact with a drop of sucrose solution, the bee expresses a reflex response by extending its mouthparts (the PER). In nature, this response allows bees to suck nectar from flowers while foraging or from a nestmate during trophallaxis. During conditioning, bees learn to associate an initially neutral odorant (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US) applied to the antennae and then to the proboscis [13, 14]. Following conditioning, bees produce a PER to the odorant alone [14, 15]. This protocol recapitulates the final phase of bees' foraging behavior, and odor-sucrose associations are thought to help bees locate and exploit rewarding floral sources.

In these experiments, bees are restrained with only their heads protruding from the holder, while their antennae and mouthparts can freely move. In the course of such an experiment, we noticed that restrained bees that were placed very close together sometimes produced a PER in absence of any other stimulation. We tested the hypothesis that such behavior may be elicited by the social stimulus represented by another worker bee. We fixed a hungry worker (the "focal bee") on one side of the workbench and progressively advanced a second, fed worker (the "stimulus bee") toward the focal bee (Figure 1A). The focal bees did not react to the presence of the stimulus bee even at a close distance of 0.5 cm. However, when the focal and the stimulus bees came in direct contact, the bees started engaging in intensive antennal movements, and a high proportion of the focal bees exhibited a PER (N = 40; Cochran test, Q = 198.29, p < 0.001; with versus without contact, McNemar, q = 32.03, p < 0.001). Thus, contact with a fed worker triggers a PER in hungry bees. Because sugar solutions naturally trigger a PER in hungry bees and the body of a fed nestmate may contain traces of sugars, several steps were taken to show that this response is indeed a social response.

The antennae of the focal bees were bathed for 15 min in zinc sulfate ($ZnSO_4$), which selectively blocks bees' contact chemosensory sensilla, impairing their gustatory detection of sugars [16]. Bees were presented with a fed nestmate and with a highly concentrated (50% w/w) sucrose solution, before and after zinc sulfate (N = 37) or solvent (N = 36) treatment (Figure 1B). In the treated group, whereas the PER to sucrose solution was severely impaired (before versus after, McNemar test, q = 22.04, p < 0.001; treatment versus control, Fisher's exact test,
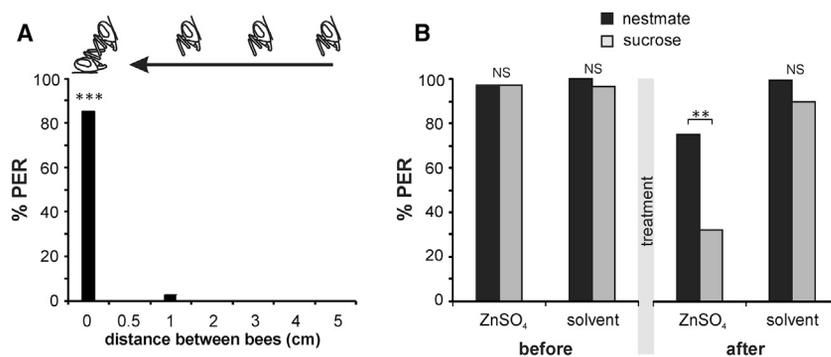
Figure 1. Social Contact Induces an Appetitive Response without Sugar Detection

(A) Percentage of starved bees extending their proboscis (%PER) to a fed nestmate according to the distance between bees. After 10 s of familiarization to the context, the focal bee faced a fed nestmate for 10 s at the indicated distance. This procedure was repeated for each distance in decreasing order with an inter-trial interval of 10 min. (N = 40, ***p < 0.001).

(B) Percentage of PER in starved bees when stimulated by a contact with a fed nestmate or with sucrose solution (50% w/w) before and after bathing both antennae for 15 min in 0.5 M $ZnSO_4$ (N = 37) or solvent (N = 31). (**p < 0.01.)

p < 0.001), responses to a nestmate were only moderately affected (before versus after, McNemar test, q = 4.90, p < 0.05; treatment versus control, Fisher's exact test, p < 0.01). Most importantly, treated individuals responded at significantly lower rate to sucrose than to a nestmate (McNemar test, q = 10.23, p < 0.01). We conclude that the PER to a nestmate does not only depend on the detection of sugar but also has a social quality through other, yet unknown, sensory cues.

Stimulation with a Nestmate Acts as an Appetitive Reinforcement

Sucrose, which naturally triggers a PER, acts as an appetitive reinforcement during olfactory learning [14]. We next asked whether stimulation with a nestmate, which triggers a PER as shown above, can likewise act as appetitive reinforcement. We thus performed standard PER conditioning experiments with the exception that the usual sucrose reinforcement was replaced by a 10 s stimulation with a nestmate (Figure 2A). We chose a differential conditioning procedure, in which one odorant (CS+) is paired with the nestmate stimulation while another odorant (CS−) is presented without this stimulus. Because odorants are always presented for 3 s before any reinforcement comes (see Figure 2A, bottom), it is possible to compare the PER to the CS+ and CS− throughout the conditioning procedure. We observed that responses to the CS+ increased in the course of conditioning (Figure 2B; N = 59; Cochran's Q test, Q = 54.5, p < 0.001), while responses to the CS− remained inexistent (Q = 0, not significant [NS]). Bees thus efficiently learned to discriminate the odorants on the basis of the social reinforcement provided (Wilcoxon test, z = 3.41, p < 0.001). In addition, this conditioning establishes stable odor-specific memories that can be retrieved 1 h later in retention tests without any social stimulus (Figure S1).

Zinc sulfate treatment had no effect at all on learning success with the social US (Figure 2C); bees from both treated and control groups increased their responses to the CS+ (treated, N = 32, Q = 26.78, p < 0.001; control, N = 33, Q = 28.91, p < 0.001) but not to the CS− (treated, Q = 4, NS; control, Q = 5, NS), and they significantly differentiated the stimuli (Wilcoxon test, treated, z = 3.41, p < 0.001; control, z = 3.72, p < 0.001). Their differentiation performance scores (the difference between responses to CS+ and CS− during the procedure, see STAR Methods) were indistinguishable (Mann-Whitney test, z = 0.22, NS). By contrast, learning was strongly impaired when sucrose

reinforcement on the antennae was used (Figure 2D). In treated bees (N = 29), responses to the CS+ reached much lower levels than in control bees (N = 31) so that differentiation scores were strongly reduced (Mann-Whitney test, z = 3.72, p < 0.001). Stimulation with a nestmate thus acts as reinforcement during appetitive olfactory conditioning through a process independent of sugar perception.

Social Reinforcement Is Mediated by Antennal Movements

In the following experiments, low-temperature melting wax was systematically applied to the mouthparts of the stimulus bee to further ensure that no sugar excretion could interfere with the experiments. We aimed to determine the sensory nature of this social reinforcement. A possible effect of visual information was excluded, since bees with both compound eyes occluded efficiently learned to differentiate the two odorants (Figure 3A; N = 37; CS+ versus CS−, Wilcoxon test, z = 4.12, p < 0.001). We asked if bees' antennae movements, which are intensive in close contact, may play a role. To address this, bees' antennal movements were blocked by using a small drop of low-temperature melting wax at their base. In the first experiment (Figures 3B and 3C), the antennae of the stimulus bee were blocked or not. This procedure hindered learning from the focal bee, as its responses neither to the CS+ nor to the CS− increased in the course of training (Figure 3B; N = 32; for both CS+ and CS−, Q = 5, NS), and no difference between stimuli appeared (Wilcoxon test, z = 0.45, NS). By contrast, when the stimulus bee's antennae were free, the focal bee learned to differentiate between CS+ and CS− (Figure 3C; N = 31; Wilcoxon test, z = 3.18, p < 0.001). These bees' differentiation scores were therefore significantly higher than in bees with blocked antennae (Mann-Whitney test, z = 3.68, p < 0.001). Importantly, this result excludes the implication of contact chemical stimuli in the social learning, because in both groups, the focal bee could touch the stimulus bee's head with her antennae and perceive this bee's cocktail of cuticular hydrocarbons. Yet, only when the stimulus bee could move her antennae did learning occur.

We then tested whether the focal bees' antennal movements are necessary to detect the social reinforcement by blocking one or both of its antennae (Figures 3D–3F). No significant differentiation between CS+ and CS− was observed when one antenna (N = 22) or both antennae (N = 23) were blocked (Wilcoxon test, z < 1.62, NS). In contrast, when the focal bees' two
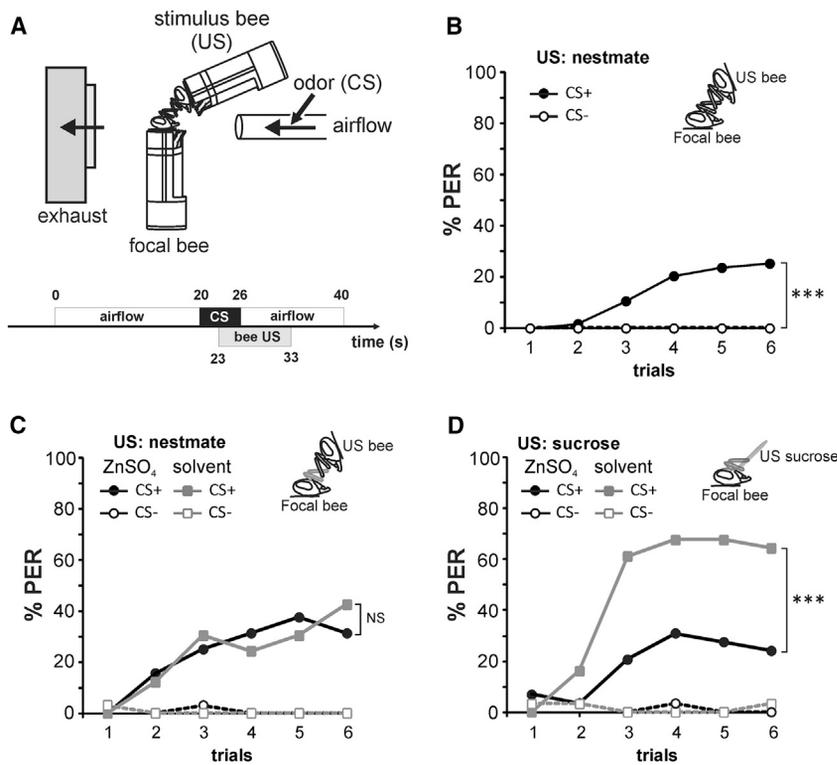
Figure 2. Social Olfactory Conditioning with a Nestmate as Reinforcement

(A) Protocol for social olfactory conditioning. The conditioned bee is placed in front of an odor delivery apparatus, before an air exhaust. At each trial, the bee remains in the permanent airflow for 20 s, then an odorant (conditioned stimulus, CS) is applied for 6 s. For the CS+, a stimulus (fed) bee (US) is brought in contact with the antennae for 10 s, starting 3 s after odor onset. After these 10 s, the bee is left for 7 s more before being removed from the apparatus. For the CS−, no other stimulation is applied. See also Figure S1 for a replication of this experiment with a 1 h memory test.

(B) Learning curves showing the percentage of bees exhibiting PER (%PER) in response to the odorant associated with a nestmate (CS+) or not (CS−) during 12 trials (6 CS+ and 6 CS− trials; N = 59, ***p < 0.001).

(C) Learning curves (%PER) for bees conditioned with a social US (nestmate) after bathing the antennae for 15 min in 0.5 M $ZnSO_4$ (N = 32) or solvent solution (N = 33). (NS, not significant.)

(D) Learning curves (%PER) for bees conditioned with a sucrose US after bathing the antennae for 15 min in 0.5 M $ZnSO_4$ (N = 29) or solvent solution (N = 31). (***p < 0.001; NS, not significant.)

antennae were free, learning occurred as normal (Figure 3F; N = 25; z = 3.18, p < 0.001). Differentiation scores were therefore significantly higher in this last group than in either group with blocked antenna(e) (Mann-Whitney test, z > 2.26, p < 0.025). These experiments suggest that social reinforcement is mediated through bees' intricate antennal communication.

Different Hive Members Bear Different Reinforcing Values

Which characteristics of a nestmate influence its quality as a social reinforcement? In honeybees, division of labor is related to age, with younger individuals remaining within the hive to perform household tasks while older individuals achieve tasks outside of the colony, such as foraging or guarding [17]. We compared the reinforcing quality of two groups of bees differing in their behavioral development: newly emerged workers (<24 h old) and mature workers that were performing outside tasks (Figure 4A). When focal bees were stimulated with a mature, outgoing worker (N = 46), conditioning was efficient, with a clear increase in responses to the CS+ (Q = 32.35, p < 0.001) but not to the CS− (Q = 5, NS) and a highly significant difference between stimuli (z = 4.01, p < 0.001). In contrast, with newly emerged bees as the US (N = 38), focal bees showed only weak performances, with only a slight increase in CS+ responses over the course of training (Q = 12.95, p < 0.05) and a generally low, although significant, differentiation between stimuli (z = 2.20, p < 0.05). Differentiation scores were highly significantly different between groups (z = 3.35, p < 0.001). Thus, different colony members, here of different behavioral developments, may have different reinforcing qualities. Since antenna movements are implied in the social reinforcement, we asked which parameters of these movements

may explain the observed difference in reinforcing quality. We used a tracking system based on a motion-capture principle to record antenna movements at a 90 Hz frequency rate [18]. Bees' antenna movements can be described in polar coordinates by a radius and an angle with the center at the antenna base (Figure 4B). We compared bees' spontaneous antenna movements for 1 min and found no difference between newly emerged (N = 25) and adult (N = 25) workers in the average position of the antennae (Figure 4B, Student's t test, radius: t = 0.85, NS; angle: t = 0.12, NS). However, the speed of antenna movements was significantly higher in adult than in newly emerged bees (Figure 4C, angular velocity: t = 2.14, p < 0.05). This suggests that the frequency of antenna contacts between the bees may support the reinforcing quality of antenna movements.

The Influence of Feeding State on the Social Reinforcement

Appetitive conditioning is known to depend on satiety state. PER probability and learning performances with sucrose as the US are generally low when the *focal* bee is satiated [19]. The same holds true for our social learning (not shown). But does the satiety state of the *stimulus* bee influence its rewarding quality? To answer this question, we compared learning performances when the social US was provided either with a starved (4 h starvation—same state as the focal bee, N = 50) or a fed nestmate (N = 56; Figure 4D). In both groups, focal bees efficiently learned to differentiate the two stimuli, with an increase in CS+ but not CS− responses (Wilcoxon test, z > 3.91, p < 0.001). No difference appeared in the differentiation scores of the two groups (Mann-Whitney test, z = 0.92, NS). In agreement with this finding,
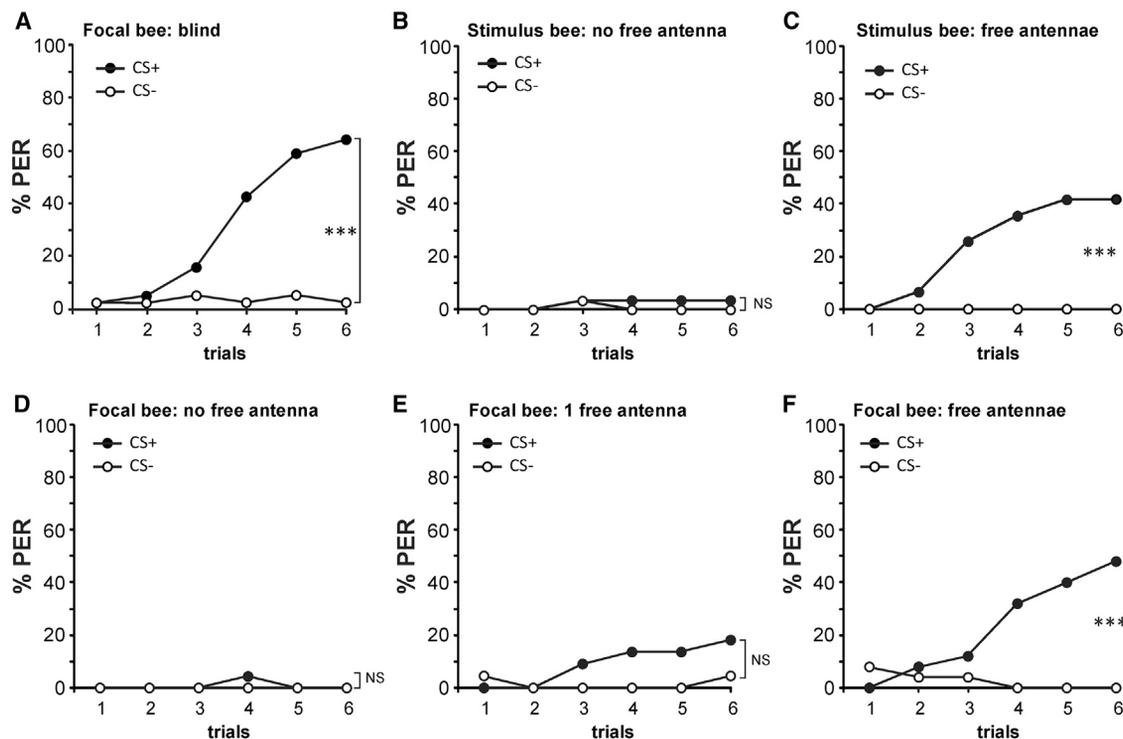
Figure 3. Social Conditioning Relies on Antennal Movements

(A) Learning curves showing the percentage of PER responses to the odorant associated with a nestmate (CS+) or not (CS−) over the course of 6 blocks of trials. The focal (conditioned) bee's eyes were lacquered to prevent the use of visual cues, and wax was applied to the mouthpart of the stimulus (US) bee to avoid any sugar excretion (N = 37, ***p < 0.001).

(B and C) Learning curves (%PER) for bees conditioned with a social US. The stimulus bee's antennae were either fixated at their base with wax (B, N = 32) or free (C, N = 31). Wax on the mouthparts of the stimulus bee precluded any sugar excretion (***p < 0.001; NS, not significant).

(D–F) Learning curves (%PER) for bees conditioned with a social US. The focal bee had both antennae fixated at their bases with wax (D, N = 23), one antenna free (E, N = 22), or both antennae free (F, N = 25). Wax on the mouthparts of the stimulus bee precluded any sugar excretion (***p < 0.001).

we found no difference between the antenna movements of starved (N = 18) and fed (N = 20) bees (Figures 4E and 4F), neither for antenna position (radius, t = 0.72, NS; angle, t = 0.90, NS) nor for antenna speed (angular velocity, t = 0.99, NS). Therefore, bees' satiety state has no influence on their antennal movements or on their reinforcing quality for other bees.

We uncovered a previously unknown form of social learning in honeybees. In restrained individuals, simple antennal contact with a nestmate triggers the extension of bees' mouthparts (PER), a behavioral response typically involved in feeding and food-exchange behaviors [11, 20–22]. The socially evoked PER is reminiscent of the behavioral sequence involved in trophallaxis, usually allowing the focal bee to taste the nectar brought by the returning forager [20]. After associating an initially neutral odorant with this social stimulus, bees start producing the PER to this odorant, exactly as they do when associating an odorant with a food reward in the canonical appetitive conditioning of the PER [13, 14]. A crucial difference, however, is that here, they do not obtain any *actual* (food) reward but only a social reinforcement. Such social learning may play an important role in the transfer of information about food sources within the hive together with previously discovered processes, like the communication of a food-source location by the waggle dance [8] and the formation of odor-nectar

associations during trophallaxis [10, 12]. Our data show that nectar transfer is not necessary for olfactory learning and that the reinforcing quality of a worker bee does not depend on its current feeding status. So, even when a returning forager has totally unloaded its crop content, it may still inform other bees about a food source's odor, thanks to its inherently rewarding value for other bees.

We investigated the reinforcing agent in this social learning and found that it involves antennal communication. In honeybees, as in most social insects, antennal contacts play an essential role in social communication and collective behaviors. Immobilization of the bees' antennae was shown to impair social interactions, aggregation, retinue behavior, and food exchanges in spite of intact senses of smell and taste [23, 24]. Tactile and vibrational cues produced during antenna contacts and sensed by antennal mechanoreceptors are thought to play a key role in these social interactions [22, 25, 26] and may be the physical agent mediating the reinforcement message.

According to current theories on social learning [5, 6], two processes may underlie our observations. First, the intrinsic reinforcing value of a nestmate may have been co-opted by natural selection in this social insect, as it is evolutionarily advantageous in a wide range of behavioral contexts, eventually affecting the
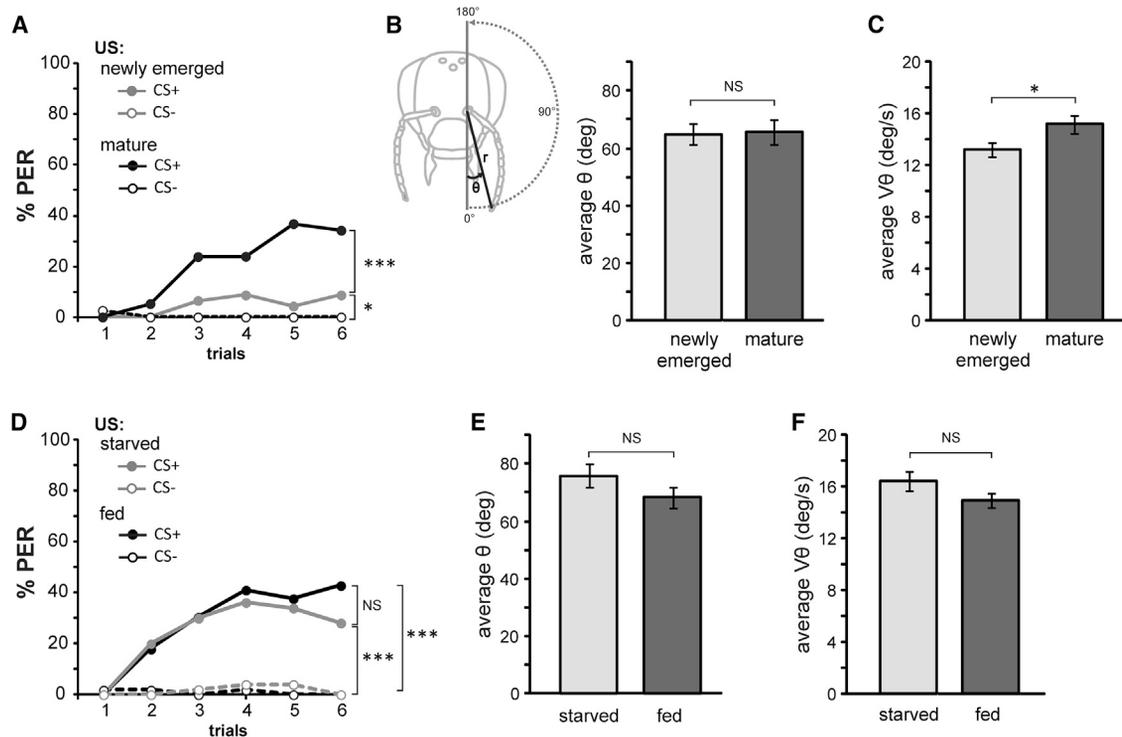
Figure 4. Social Conditioning Depends on the Physiological Status of the Stimulus Bee

(A) Learning curves (%PER) for bees conditioned with a social US. The stimulus bee was either a mature outgoing bee (same age as the focal bee, N = 38) or a newly emerged bee (N = 46). Wax on the mouthparts of the stimulus bee precluded any sugar excretion (***p < 0.001; *p < 0.05).

(B and C) Measure of antenna position and velocity in bees of different ages, using a camera to detect colored dots on bees' antenna tips. Left: polar coordinates were used. An antenna's angular position (θ, in degrees) was defined as the angle between a line connecting the antenna tip to its base (black) and an anteroposterior line passing through the antenna base (dark gray). The angular velocity (Vθ) was calculated as the angle θ traveled by each antenna per second. Right: average angular position (B) and angular velocity (C) during 1 min recordings (mean ± SEM) according to the bees' ages (newly emerged bees, N = 25; mature bees, N = 25; *p < 0.05).

(D) Learning curves (%PER) for bees conditioned with a social US. The stimulus bee was either fed *ad libitum* (N = 56) or starved for 4 h (N = 50) prior to the experiment. Wax on the mouthparts of the stimulus (US) bee precluded any sugar excretion (***p < 0.001).

(E and F) Average angular position (E) and angular velocity (F) of bees' antennae during 1 min recordings (mean ± SEM) according to their satiety level (starved, N = 18; fed, N = 20).

fitness of sexuals. Alternatively, it may be acquired during each bee's lifespan. The associative learning phenomenon of *second-order conditioning* has been repeatedly evoked to explain social learning in insects [3, 5, 27]. In short, if an animal first learns an association between a CS1 and a US and experiences a subsequent association between a new CS2 and the CS1, then the CS2 becomes a predictor of CS1 and indirectly of the US. Here, adult workers have spent their entire life in the hive, receiving food (US) following antennal contacts with a nestmate (CS1). In our assay, they would learn a second-order association so that a neutral odorant (CS2) becomes a predictor of nestmates (CS1) and indirectly of food (US). Deciding between both possibilities will require rearing focal and/or stimulus bees in total absence of any interactions with conspecifics. Remarkably, acquisition was generally slower, and the asymptotic level of performances was lower than in usual odor-sucrose PER learning [13, 14, 18]. This could mean that experimental conditions are not optimal for social learning (in particular, restraining may render fine antennal communication difficult) or that the social US is indeed weaker, possibly through the second-order learning process. Note, however, that several other

associative conditioning paradigms in restrained honeybees also produce such limited acquisition success (aversive conditioning to odors [28–31] and colors [32]; appetitive conditioning to colors [33, 34]).

In the bee brain, sucrose reinforcement is thought to be mediated by a single octopaminergic neuron, VUM-mx1 [35]. Blocking octopaminergic neurotransmission typically impairs PER conditioning [36], and future work should evaluate whether this is the case for the social learning. If so, one will have to understand whether this is due to the second-order mechanism presented above or if different forms of ''appetitive'' US all feed onto the same reinforcement pathways, here possibly VUM-mx1. Alternatively, social learning may use an independent reinforcement pathway, which will need to be determined.

These findings provide new elements to understand how information exchanges within a social group may support efficient collective behavior and optimal resource exploitation. Because this social conditioning protocol on restrained individuals can be coupled with invasive techniques (pharmacology, electrophysiology, or optical imaging), the neurophysiological correlates of social learning now appear within reach.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - $ZnSO_4$ treatment for blocking antennal detection of sugar
  - Preventing regurgitation
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  - Antenna monitoring apparatus
- QUANTIFICATION AND STATISTICAL ANALYSIS
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  - Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.03.025.

AUTHOR CONTRIBUTIONS

H.C. participated in study design, ran experiments and data analyses, participated in figure design, drafted the manuscript, and contributed to the final version. J.C. participated in study design, oversaw initial experiments, and provided critical input to the final manuscript. S.F. and H.M. performed experiments and analyses. G.A. participated in study design and provided critical discussion on the final manuscript. J.C.S. led the study and oversaw figure design, data analyses, and manuscript writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
ZnSO4	Sigma Aldrich	83265-250ML-F
1-hexanol 99%	Sigma Aldrich	471402-100ML
1-nonanol 99%	Sigma Aldrich	74280-100ML
Experimental Models: Organisms		
Honeybee Apis mellifera	CNRS Gif-sur-Yvette	N/A
Software and Algorithms		
Brain Vision Systems	http://www.bvs-tech.com/website/eng/index.php	BIPcam
Statistica®	StatSoft, Inc. 2004	Statistica 7.0
Other		
Posca pen	https://www.mba-shop.com	Posca PC-5M, Mitsubishi Pencil Co.
White light source	Leica, Jena, Germany	CLS 150XE
Low-temperature melting wax	www.kerrdental.com/kerr-laboratory/utility-wax-waxes	09731

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jean-Christophe Sandoz (sandoz@egce.cnrs-gif.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Honeybee workers (*Apis mellifera*) were caught at the hive entrance on the CNRS campus of Gif-sur-Yvette. In one experiment, newly emerged bees were used as stimulus (US) bee (Experiments in Figures 4A–4C). A brood frame was taken from the hive on the day prior to the experiment and placed in an incubator (at 35°C). Individual workers were caught on the day of the experiment when emerging from the cells.

METHOD DETAILS

Subject's preparation
Bees were chilled on ice until they stopped moving and were harnessed individually in plastic holders, leaving their antennae and mouthparts free. Depending on the season and state of the hive at the moment of the experiment, the bees where either placed in a plexiglas cage [37] the day before the experiment, providing honey and water *ad libitum* for 4 hours, before being harnessed for the night and fed on the morning with 2 μl of sugar solution (50% w/w) (experiments in Figures 1A, 2B, 3B, 3C, and 4A–4F), or were caught directly on the morning of the experiment, harnessed and fed with 5 μl of sugar solution (50% w/w) (experiments in Figures 1B, 2C, 2D, 3A, 3D–3F, and S1). These conditions were ideal to obtain highly motivated focal bees when the experiments started 3-4 h afterward. Only bees that displayed PER to 50% w/w sucrose in the morning feeding were kept for the experiments. Contrary to the focal bees, the stimulus bees were fed *ad libitum* in the morning, except when explicitly mentioned (starved US nestmates, Experiment 4D-F). Social conditioning experiments were always carried out with focal and stimulus bees obtained from the same colony.

Responses to social contact
The proboscis extension response (PER) of the focal bee was measured in response to the manual approach of a stimulus bee. At each trial, after 10 s of habituation to the setup, the bee was approached and faced the nestmate for 10 s, before being removed. The occurrence or not of a PER in the focal bee during this period was scored as 1 or 0 respectively. This procedure was repeated for all tested distances, with an inter-trial interval of 10 min. At each trial the distance was reduced: 5 cm, 4 cm, 3 cm, 2 cm, 1 cm, 0.5cm and then contact (0 cm), allowing both bees to touch each other with their antennae.

PER conditioning with a social US
Conditioning of the proboscis extension response (PER) was carried out in standard conditions [14, 38]. In the case of social conditioning, the unconditioned stimulus (US) was the presentation of a nestmate (stimulus bee) approached to the head of the focal bee

for 10 s, allowing contact between their antennae. Throughout conditioning, one focal bee was always stimulated with the same stimulus bee. One stimulus bee served as US for 3 focal bees.

All bees received a differential conditioning procedure in which one odorant (CS+) was associated with the social US (i.e., reinforced) and another odorant (CS−) was presented explicitly without US (i.e., non-reinforced). Such a protocol contains an internal control, as animals that efficiently learned the CS–US association will respond to the CS+ but not to the CS− [38]. The CSs were 5 mL of pure odorant (1-hexanol or 1-nonanol) delivered to the antennae of the bee at a distance of 2 cm for 6 s, using an olfactory stimulation device or syringes (experiments in Figures 3A, 3D–3F, and S1).

The olfactory stimulation apparatus was connected to a pump, enabling the constant circulation of an air flow of 52.5 mL/sec. This flow, composed of a principal air flow of 50 mL/sec and a secondary flow of 2.5 mL/sec, was directed to the bee by a glass tube (0.5 cm diameter), at a distance of 2 cm. The secondary airflow could be directed to one of two subcircuits (one containing an odorant source, and another without any odorant) before being reinjected into the main airflow. Most of the time, air flowed through the odorless subcircuit. Olfactory stimulation was applied manually inducing a switch of the secondary flow to the odorant subcircuit for 6 s. The odorant subcircuit included a Pasteur pipette containing a piece of filter paper (20 × 2 mm) soaked with 5 mL of odorant solution. The other subcircuit included an identical Pasteur pipette without odorant. An air extractor, placed behind the bee, prevented odorant accumulation.

Each day, half of the individuals received 1-hexanol (A) reinforced and 1-nonanol (B) non-reinforced, and vice versa for the other half of the bees. Conditioning consisted of 12 trials (6 CS+, 6 CS−) with an inter-trial interval of 10 min. Odorants were presented in a pseudo-random sequence of six reinforced and six non-reinforced trials (ABBA BAAB ABBA) starting with the odorant A or B in a balanced manner, so that no effect of a particular odorant could influence the results. Each conditioning trial lasted 40 s (20 s of airflow, 6 s of olfactory stimulation, and 14 s of airflow). Each individual was placed on the stimulation site, under a cold light source, in front of the air extractor to prevent odorant accumulation. In the case of the CS+, the social US was applied 3 s after odorant onset, for 10 s. This long duration for the US was chosen because in preliminary trials we observed that the focal and the stimulus bees engaged in antennal communication before the focal bee exhibited PER, and typically PER was elicited more slowly than with a sucrose stimulus. In all experiments, PER responses to the CSs were measured during the 3 s in which the bees were exposed to the odor only (before any US presentation).

In some experiments, PER conditioning was performed using a sugar US instead of a social US. The protocol was identical except that the US was a 50% (w/w) sucrose solution delivered for 3 s to both antennae. No sugar was delivered to the proboscis of the bees and no actual sucrose reward was given.

ZnSO$_4$ treatment for blocking antennal detection of sugar

To ensure that PER in response to a nestmate was not due to sugar detection, a treatment with ZnSO$_4$ which is known to specifically block PER to sugar stimulation on the antennae was applied [16]. The contact chemoreceptors thought to be blocked by this treatment are the *sensilla trichodea* D [16, 39]. The antennae of each bee were inserted into two capillary tubes, which were then filled with a 0.3% Triton X solution containing 0.5M ZnSO$_4$ (Sigma Aldrich) for treated bees or 0.3% Triton X for solvent controls. The treatment was applied for 15 min.

PER was first measured in response to a 10 s social contact, and then in response to 3 s sugar stimulation, with an inter-trial interval of 10 min. Then ZnSO4 or control treatment were applied. One hour after treatment, the social and sugar stimulations were tested again. In another experiment we evaluated the effect of ZnSO$_4$ treatment on social and appetitive conditioning (with sugar US). The conditioning experiments started 1 hour after the end of the treatment.

Preventing regurgitation

In all experiments, except the initial one (1A) and those testing the effect of ZnSO4 treatment (1B, 2B-D), low-temperature melting wax was applied on the mouthparts of the stimulus bees to avoid any sugar solution regurgitation during the experiment.

Blocking antennal movements

In some experiments, the focal or the stimulus bees' antennal movements were blocked. Low-temperature melting wax was applied on the socket (base) of each antenna, to block the scape in position. In this way, any movements of the antennae around their base were blocked, but the flagellum was not impacted and the bees' olfactory modality remained intact. This bee's antennae were placed in contact with those of the other bee during the social US but antenna movements were blocked.

Antenna monitoring apparatus

The recording apparatus was composed of a camera positioned above the bee holder. The camera included an integrated processing card allowing adaptive detection (using a motion prediction algorithm) of the two color dots painted on the two last flagellomeres of the antenna, up to a rate of 120 Hz (BIPcam, Brain Vision Systems). The camera recorded the coordinates of the two color dots on the antenna tips, in real time at a rate of 90 Hz. In order to optimize the detection of the color dots, the apparatus was placed in low light conditions (controlled and kept constant). A cold light illumination ring was placed around the lens of the camera, diffusing homogeneous white light on the bee's head (Leica CLS 150XE, Leica, Jena, Germany). The intensity of the light source was tuned precisely to allow optimal detection and kept constant for the duration of the experiments.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of antennal movement recordings

Before the recording period, each bee was left to acclimatize to the setup for 20 s. Each recording lasted 60 s. The monitoring apparatus [18] recorded at each time point the location of the two antenna tips of each bee on the camera sensor. First, all the recordings from all bees were recalculated in the same coordinate system (x,y), with the socket of the right antenna as the origin (coordinate 0,0) and the socket of the left antenna as the unit reference on the x axis (coordinate 1,0). Each recording thus resulted in a series of (x,y) coordinates for each antenna at each time-step (1/90 s). This allowed a comparison between the antennal movements of different bees. Bees' antennal movements are best described using circular coordinates (r, θ), as each antenna moves around its socket [18] (Figure 4B). Thus, each antenna's movements were described in their own coordinate system, with the antenna socket (base) as the origin (0,0).

- **Angular position (θ):** it was defined as the angle between a line connecting each antenna tip to its base (r) and an anteroposterior line passing through the corresponding antenna base. This variable indicates if the antenna is positioned to the front (0°), to the side (90°) or backward (180°). Note that the measured angle is symmetrical for the left or the right antenna so that 90° is on the left for the left antenna and on the right for the right antenna.
- **Angular velocity (Vθ):** it was calculated as the angle θ traveled by each antenna during a frame (1/90 s). It is expressed in degrees per second.

Statistical analysis

Differences between bees' PER to sugar solution and to a nestmate were compared using a McNemar test. Fisher's exact tests were used to compare PER between treatment groups ($ZnSO_4$ versus solvent). In the conditioning experiments, changes in PER to the CS+ or to the CS− in the course of training were analyzed using Cochran's Q tests. Differences between the numbers of responses to the CS+ and to the CS− were analyzed using Wilcoxon tests. In each group, a differentiation performance score was calculated as the number of responses to the CS+ minus the number of responses to the CS−. Comparisons of these scores between groups were performed using Mann-Whitney tests. Spontaneous antennal movement (angle and velocity) were compared between groups using Student's t test. All statistical analyses were performed with Statistica 7.0 (StatSoft, Inc. 2004).

Supplemental Information

Social Contact Acts as Appetitive Reinforcement

and Supports Associative Learning in Honeybees

Hanna Cholé, Julie Carcaud, Hélène Mazeau, Sylvain Famié, Gérard Arnold, and Jean-Christophe Sandoz
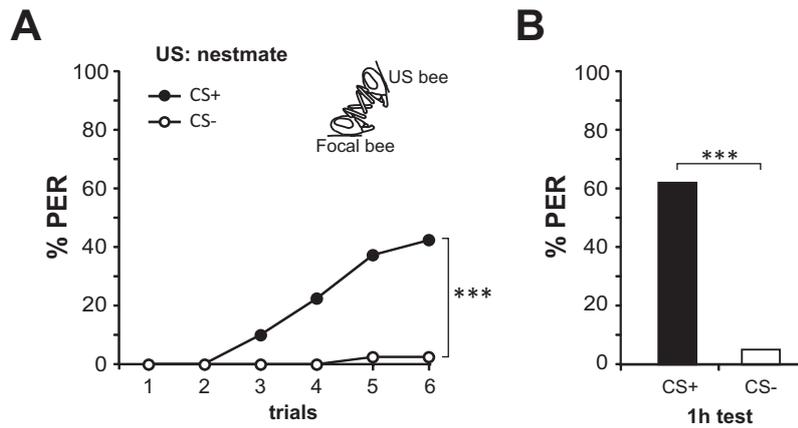
Figure S1: Memory of the odor-nestmate association. Related to Figure 2.
A) Learning curves showing the percentage of bees exhibiting PER (%PER) in response to the odorant associated with a nestmate (CS+) or not (CS-) during 12 trials (6 CS+ and 6 CS- trials; N=40 ***: p<0.001, RM-ANOVA). B) Performances in memory tests with the CS+ and CS- performed 1h after conditioning (***: p <0.001, Mc Nemar test).