

as 100 unit cells (37), but in the solid state, the mean free path of electrons in semiconductor 1D superlattices has been limited to 10 unit cells (38). Our experiment elucidates the key features of miniband electron dynamics in a moiré superlattice and points toward further explorations of novel transport effects. For instance, the saddle-point VHS could host exotic effects caused by enhanced electron-electron interactions (19, 39), and valley-contrasting physics could be accessed by taking advantage of the severe trigonal warping of minibands (40). For technology, such a clear validation of the miniband conduction properties suggests that graphene/h-BN (and perhaps other moiré superlattices) may be a practical platform for devices based on miniband physics. Efficient photocurrent generation at the edge of a graphene superlattice in a magnetic field (41) may be caused by the skipping orbits we have observed; furthermore, THz devices such as the Bloch oscillator can benefit from the much longer scattering times in this system.

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## SUPPLEMENTARY MATERIALS

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## COGNITION

# Unexpected rewards induce dopamine-dependent positive emotion-like state changes in bumblebees

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Whether invertebrates exhibit positive emotion-like states and what mechanisms underlie such states remain poorly understood. We demonstrate that bumblebees exhibit dopamine-dependent positive emotion-like states across behavioral contexts. After training with one rewarding and one unrewarding cue, bees that received pretest sucrose responded in a positive manner toward ambiguous cues. In a second experiment, pretest consumption of sucrose solution resulted in a shorter time to reinitiate foraging after a simulated predator attack. These behavioral changes were abolished with topical application of the dopamine antagonist fluphenazine. Further experiments established that pretest sucrose does not simply cause bees to become more exploratory. Our findings present a new opportunity for understanding the fundamental neural elements of emotions and may alter the view of how emotion states affect decision-making in animals.

Emotions are transient subjective states, underpinned by physiological, behavioral, and cognitive phenomena, triggered by appraisal of environmental situations (1–3). Our conceptual understanding of emotions is largely based on human subjective experiences—what we “feel”—assessed directly through verbal reports. In animals, similar emotion-like states can be inferred through observable, quantifiable parameters. To ensure that the criteria of emotion-like states are met and to distinguish these from other forms of environmentally induced states, perhaps driven by learning, we must quantify the range of physiological, behavioral, and cognitive phenomena that occur in response to environmental factors, similar to those studied in humans (4).

The majority of work on animal emotions focuses on mammals and almost exclusively on negative emotions (5). The idea that invertebrates may exhibit basic forms of emotion is increasingly accepted (6–8), and given the assumed adaptive function of emotions [to coordinate the individual's cognitive and behavioral resources toward fitness-relevant priorities (1, 2, 9)], we might expect that a diversity of emotion-like states, includ-

ing positive ones, exist across phyla, albeit not necessarily consciously so (9–11).

In humans, consumption of sweet snacks can induce positive emotions (12–14). Here we examine whether consuming a small amount of sucrose solution before performing a test causes bumblebees (*Bombus terrestris*) to behave in a way that is indicative of an induced positive emotion-like state.

In experiment 1.1, we used the well-established judgment bias paradigm, in which subjects associate one cue with a positive event and another cue with a negative event (15). Subjects in a positive emotion state tend to respond to ambiguous (intermediate) stimuli as though predicting the positive event (4).

We trained bees in a go/no-go task. On some trials, bees learned to enter a cylinder beneath a colored (e.g., blue) placard on one side of an arena, where they would find a 30% sucrose solution

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(Fig. 1, A and B). On alternate trials, bees learned not to enter a cylinder at the opposite side of the arena under a placard of different color (e.g., green), where they would find no reward (water only). The latency from the time that bees entered the arena to the time that they entered the presented cylinder was recorded.

We then examined bees' response to ("judgment" of) ambiguous information (intermediate placard color and position; Fig. 1C). Half of the trained bees, randomly selected, received for the first time a 5- $\mu$ l droplet (equivalent to <5% of stomach capacity) of 60% sucrose solution in the tunnel leading to the arena; the other half received no pretest reward. Bees that consumed sucrose solution before making a decision took less time to enter the chamber of the middle ambiguous stimulus (Fig. 1D, tables S1 and S2, and supplementary materials).

Could it be that when bees consumed the small pretest reward, rather than experiencing a positive emotion-like state, a higher expectation of subsequent reward resulted in greater exploration of novel stimuli? Previous work indicates that honeybees' foraging choices are controlled by short-term memories initiated by recently experienced rewards (16, 17). However, in our study, bees tested with stimuli that were not intermediate to the trained stimuli (novel in terms of color, position, and number; experiment 1.2; Fig. 1E) exhibited no difference in choice time (Fig. 1F and table S3) or number of choices (Fig. 1G and table S4) between the two groups, indicating that pre-decision sucrose consumption did not cause a general increase in expectation of reward.

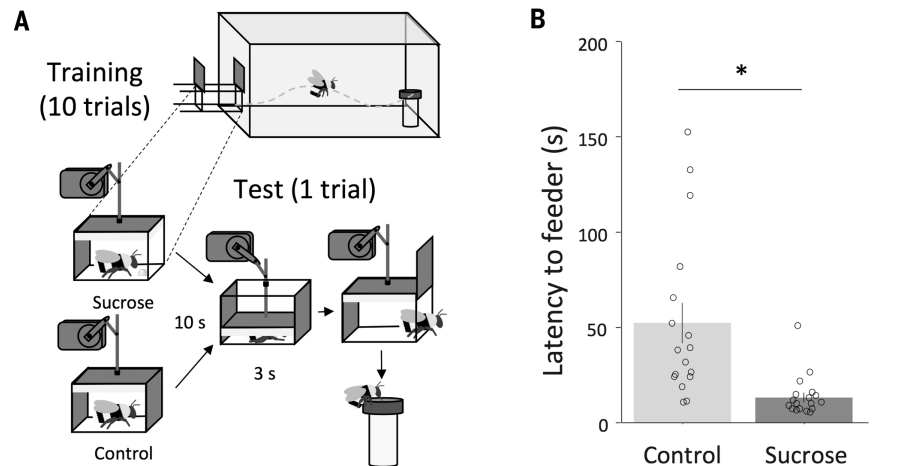
We considered whether the consumption of sucrose solution may simply make bees more excited or active, resulting in faster decisions in response to ambiguous stimuli. Thorax temperature increased after consumption of 5  $\mu$ l of 60% sucrose solution ( $n = 72$ ,  $t_{70} = 6.78$ ,  $P = 3.12 \times 10^{-9}$ ; experiment 2.1; fig. S1, A and B, and supplemental materials), denoting increased metabolic rate. But this did not translate to increased activity. Sucrose-receiving and control bees ( $n = 12$  per group) showed no difference in flight time ( $t_{22} = 0.666$ ,  $P = 0.512$ ) or speed ( $t_{22} = 0.241$ ,  $P = 0.812$ ) to reach a feeder (experiment 2.2; fig. S1, C and D, and supplemental materials), and when the feeder was removed, speed during a 120-s flight also did not differ between groups ( $n = 24$ ,  $t_{22} = -0.403$ ,  $P = 0.691$ ; experiment 2.3; fig. S1E), suggesting that unexpected rewards did not affect bees' overall activity level.

It has been argued that one characteristic of emotions across species is generalization, a property whereby an induced emotion state operates across behavioral contexts (9). To examine whether these behavioral results were similar across contexts, we tested whether an unanticipated reward would change bees' reaction to later aversive stimuli (experiment 3). We trained bees to forage at a feeder containing 30% sucrose solution. After training and on their next foraging trip, bees were held temporarily in the tunnel connecting the hive and arena. Bees either received an unanticipated 5- $\mu$ l droplet of 60% sucrose solution

### Fig. 1. Judgment bias in response to ambiguous stimuli.

(A) Set up for experiment 1.1. (B and C) Each row shows a "bee's-eye view" of placards within the arena. Training stimuli for one of four counterbalanced orientations are shown in (B) (N, negative stimulus position, P, positive stimulus position; fig. S2). Bees ( $n = 24$ ) were trained to find sucrose solution in a cylinder under one placard and to avoid another. Only one cylinder was accessible in any one trial; odd trials were rewarded and even trials unrewarded. The testing procedure is shown in (C) (NP, near the positive stimulus position; M, middle position; NN, near the negative stimulus position). Half of the bees received pretest sucrose (arrowheads). After two "reminder" trials, bees

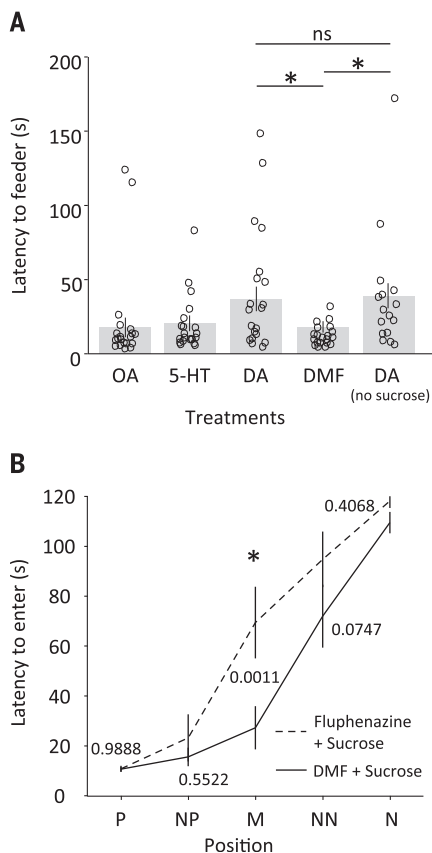
were tested with three ambiguous stimuli that alternated between the trained stimuli. The order was counterbalanced (fig. S3). (D) Results of experiment 1.1. The sucrose-receiving group took less time to enter the middle position (M) than the control group. Numbers shown are  $P$  values (the asterisk indicates significance). (E) Training procedure for experiment 1.2 [the view is as in (B) and (C)]. Bees ( $n = 24$ ) were trained to find a reward under a blue placard and subsequently tested with two novel stimuli. (F and G) Results of experiment 1.2. Latency time to the feeder (F) and the number of choices (G) did not differ between groups. Here and elsewhere, bars indicate means, open circles represent individual bees, and error bars denote standard error. Generalized linear modeling analyses are reported in tables S1 to S4.



**Fig. 2. Attenuation of response to aversive stimuli.** (A) Training and test procedure for experiment 3. Bees ( $n = 35$ ) were trained to feed at a 30% sucrose solution feeder. Subsequently, bees received or did not receive 5  $\mu$ l of 60% sucrose solution before a simulated predator attack. (B) Results of experiment 3. Sucrose-receiving bees took less time to resume foraging behavior than the control group ( $t_{33} = -3.70$ ,  $*P = 7.87 \times 10^{-4}$ ).

or nothing (control). After a 10-s delay, a predator attack was simulated. In nature, bees are sometimes ambushed at flowers by sit-and-wait predators such as crab spiders; bees often escape after a brief struggle, allowing them to modify

their subsequent behavior to cope with such threats (18). Mimicking such an attack, each bee was captured by a trapping mechanism, in which constant pressure was applied for 3 s by a stamp-shaped device softened with a sponge and connected to



**Fig. 3. Results of experiments blocking biogenic amines.** All bees received 5  $\mu$ l of 60% sucrose solution before testing, except as indicated in the right-most column of (A). (A) Results of experiment 4.1. Dopamine (DA) antagonist-treated bees—but not octopamine (OA) antagonist-treated bees, serotonin (5-HT) antagonist-treated bees, or DA antagonist-treated bees that did not receive pretest sucrose—took more time to resume foraging behavior than DMF-treated bees after a simulated predator attack (asterisks denote significance; ns, not significant). (B) Results of experiment 4.2. Fluphenazine (DA antagonist)-treated bees took more time to enter the middle position (M) than DMF-treated bees, indicating that drug treatment inhibited the judgment bias caused by pretest sucrose in the control group. Numbers shown are *P* values (the asterisk indicates significance). Generalized linear modeling analyses are reported in tables S5 to S6.

a micro-servo (Fig. 2A) (18). The bee was subsequently released, and the time it took to commence foraging was recorded.

Sweet food can increase positive emotions and improve negative mood in human adults, and reduce crying and grimacing of newborns in response to aversive stimuli (12–14). If drinking an unexpected sucrose solution caused a positive emotion-like state in bees, we predicted that, after consumption, bees' aversive reaction to the "predator" would be attenuated. Indeed, bees that consumed sucrose solution before the "attack" took less time to reinstate foraging ( $n = 35$ ,  $t_{33} = -3.70$ ,  $P = 7.87 \times 10^{-4}$ ; Fig. 2B).

The insect reward system parallels that of mammals in several aspects, including some of the neurochemicals involved (19). In mammals, several neurotransmitters play key roles in both reward processing and emotions. We asked whether the biogenic amines linked to reward processing in the insect brain might be involved in the behaviors suggestive of emotion-like states that we observed. We topically treated bees (20, 21) with antagonists of the biogenic amines octopamine (OA; antagonist, mianserin;  $n = 20$ ), dopamine (DA; antagonist, fluphenazine;  $n = 20$ ), and serotonin (5-HT; antagonist, yohimbine;  $n = 20$ ) and determined their effect on the behavior induced by pretest sucrose. Bees were trained as in experiment 3. Fifteen minutes after application of an antagonist or vehicle control (*N,N*'-dimethylformamide, DMF;  $n = 20$ ), bees received, for the first time, a 5- $\mu$ l droplet of 60% sucrose solution. After this, bees were subjected to a simulated predator attack, and the time taken to return to foraging was recorded (experiment 4.1). Only bees treated with the DA antagonist took longer to begin foraging than control bees (analysis of variance,  $n = 96$ ,  $F_{4,90} = 3.48$ ,  $P = 0.011$ ; Tukey post hoc test,  $P = 0.039$ ; Fig. 3A). We speculate that this is a consequence of brain DA signals responding to an unexpected reward (22–25). To ensure that the DA antagonist was not simply interacting with pathways mediating normal response to the aversive stimulus, bees were topically treated with DA antagonist without receiving pretest sucrose. The time to begin foraging for these bees was similar to both that of bees treated with DA antagonist and given pretest sucrose and that of control bees given no pretest sucrose [ $n = 16$ ; Figs. 3A (DA antagonist and pretest sucrose) and 2B (control)].

We explored whether blocking DA had similar effects on the observed cognitive consequences of pre-decision reward in the judgment bias paradigm. Bees were trained as in experiment 1.1 and then treated with either DA antagonist or DMF 15 min before consuming an unexpected 5  $\mu$ l of 60% sucrose solution and entering the test arena. Compared with control bees, DA antagonist-treated bees took longer to enter the middle ambiguous stimulus chamber (experiment 4.2; Fig. 3B and tables S5 and S6).

Recent evidence suggests clear roles for DA in reward-related processes in invertebrates (23), including motivation for reward (25), nutritional valuation of reward (22), and arousal (26). Our results corroborate DA's role in the neuronal processes mediating reward signals in bees. An intriguing prospect for research would be whether similar circuits controlling wanting, hunger, nutritional valuation, and/or arousal underpin the emotion-like states in bees indicated by our results.

The behaviors displayed by bumblebees in response to a small amount of pre-decision sucrose conform to the criteria commonly applied to mammals for the interaction of internal emotion-like states with decision-making—namely, positive judgment bias in response to ambiguous stimuli and attenuated response to negative stimuli. Whether common neural processing features evolved independently or an ancient role of biogenic amines

evolved to serve similar functions, new findings (including ours) support the hypothesis that the fundamental elements of emotion exist in many species (9).

Our results lend support to the notion that invertebrates have states that fit the criteria defining emotion (1, 9). The adaptive function of emotions is thought to be the integration of information about the environment and body to modulate decisions and behavior (9). Understanding and investigating the basic features of emotion states will bring us a step closer to determining the brain mechanisms underlying emotion across taxa.

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## SUPPLEMENTARY MATERIALS

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