Report

Current Biology

An inhibitory signal associated with danger reduces honeybee dopamine levels

Highlights

- Receiving an inhibitory stop signal can decrease bee head dopamine levels
- Receiving a stop signal can therefore depress the hedonic value of food
- Increasing dopamine decreased bee stop signaling and time spent in the hive
- Increasing brain dopamine increased the time spent feeding and waggle dancing

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In brief

Dong et al. demonstrate that an inhibitory signal, the stop signal, which counters waggle dancing and is triggered by negative events at the food source, can also decrease head dopamine levels and dancing, even if the dancer had no negative experiences. Increasing the brain dopamine levels reduced the aversive effects of an attack.

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An inhibitory signal associated with danger reduces honeybee dopamine levels

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SUMMARY

Positive and negative experiences can alter animal brain dopamine levels.[1] When first arriving at a rewarding food source or beginning to waggle dance and recruit nestmates to food, honeybees have increased brain dopamine levels, indicating a desire for food.[2] We provide the first evidence that an inhibitory signal, the stop signal, which counters waggle dancing and is triggered by negative events at the food source, can decrease head dopamine levels and dancing, independent of the dancer having any negative experiences. The hedonic value of food can therefore be depressed simply by the receipt of an inhibitory signal. Increasing the brain dopamine levels reduced the aversive effects of an attack, increasing the time that bees spent subsequently feeding and waggle dancing and decreasing their stop signaling and time spent in the hive. Because honeybees regulate food recruitment and its inhibition at the colony level, these results highlight the complex integration of colony information with a basic and highly conserved neural mechanism in mammals and insects.[2]

RESULTS AND DISCUSSION

In multiple animals, dopamine is involved in arousal, cognition, and sensitivity to stimuli.[1] Dopamine is also associated with seeking and wanting behavior, particularly with the pleasurable experiences of reward. Recently, Huang et al.[2] demonstrated that honeybee (*Apis mellifera*) brain dopamine levels transiently increased when bees arrived at a profitable food source and when they began waggle dancing to communicate food location and recruit nestmates. Higher bee hunger levels also increased brain dopamine levels, enhanced the perceived value of sucrose solutions, and improved rewarded olfactory learning and memory. Honeybees fed dopamine followed waggle dancers 15% longer than control bees, suggesting that dopamine increased their interest in obtaining food location information, enhanced food seeking, or both.[3] Older bees (foragers) also have higher brain dopamine levels than younger bees working inside the hive,[4,5] demonstrating another link with food wanting. Like vertebrates, honeybee dopamine brain levels are thus also associated with a hedonic value system of wanting.[2]

The perceived value of a food source is complex and associated with multiple factors beyond its net caloric value for any given individual—colony needs also matter.[6] Predators can significantly reduce the perceived value of a food source and therefore reduce waggle dancing for a dangerous resource.[7] Honeybees therefore have a sophisticated mechanism to communicate peril. To counteract the positive feedback generated by the waggle dance, which increases the number of nestmates exploiting a food source, *A. mellifera* and *Apis cerana* possess an inhibitory signal, the stop signal, which is targeted at dancers advertising a dangerous or declining food source.[8,9] The stop signal is a vibratory signal produced when the signaler has a negative experience with food—being attacked by a predator[8,10] or conspecifics, or experiencing an overcrowded resource.[9] Stop signals are generally delivered by the signaler butting its head against a waggle dancer, which causes the waggle dancer to perform fewer dance circuits, thereby reducing recruitment to a now dangerous or over-exploited food source.[8] The stop signal can also be an alarm signal, galvanizing appropriate colony defensive actions.[7,11]

Studies of the co-evolutionary arms race between hornets and their honeybee prey have yielded multiple insights.[12] The giant hornet, *Vespa mandarinia*, is a formidable predator and attacks multiple species of honeybees in its native range in Asia.[13,14] This hornet has also recently invaded North America, where it poses a serious threat given that *A. mellifera* and native bee species have not co-evolved with it and have largely ineffective defenses.[15,16] A few workers of *V. mandarinia* can exterminate a large *A. mellifera* colony of up to 30,000 individuals within a day.[17] Honeybees thus avoid these hornets at food and exhibit aversive behavior that could correspond to fear,[18] although it is unclear how to best measure such negative affect. A potentially useful entry point is to consider how predators, or the warning signals that they engender, affect the honeybee wanting system.

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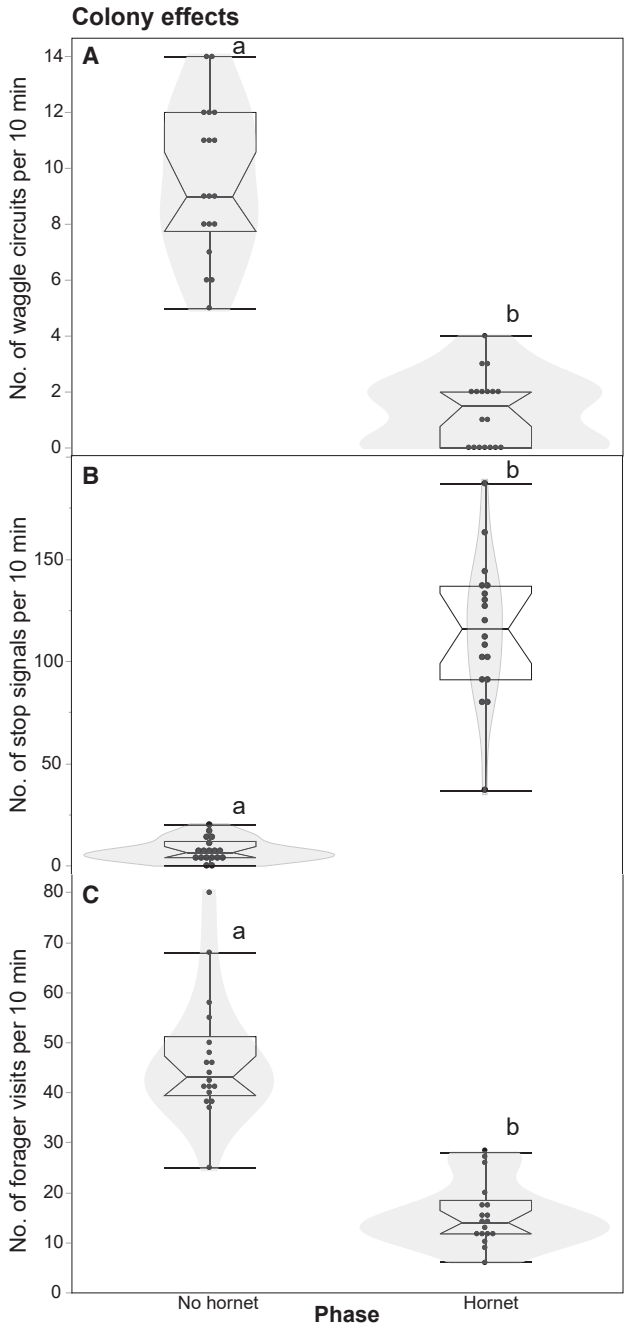

Figure 1. Hornet attacks decreased waggle dancing and increased stop signaling at the colony level

(A) Hornet attacks at the feeder decreased colony-level waggle dancing because most dances were for the feeder, (B) increased stop signaling, and (C) decreased the number of forager visits to the feeder. Different letters indicate significant differences ($p < 0.0001$). Notched box plots, violin plots, and the actual data (black-filled circles) are shown for all figures. In this experiment, we conducted 18 trials with no hornet and 18 trials with hornet attacks.

The recent discovery that dopamine is associated with honeybee food wanting[2] raises the possibility that a signal about danger at a food source can, by itself, decrease foraging motivation and thus reduce brain dopamine levels. Dopamine is involved in negative-valence signaling and, as part of its hedonic role in learning, is required for fear conditioning in mice, rats,[19,20] and honeybees.[21]

We hypothesized that predation would counteract forager food wanting, resulting in them giving up foraging and staying inside the hive with a concomitant decrease in bee head dopamine levels. We predicted that the normal transitory increase in dopamine in waggle dancers would be eliminated by their receipt of stop signals. We also hypothesized that artificially increasing dopamine levels by feeding bees dopamine would reduce the aversive effects of hornet attacks.

Bees from three *A. mellifera* observation colonies were trained to 50% w/v sucrose solution feeders for 30 min and then subjected to two phases: no attack or attack by a tethered *V. mandarinia* hornet.[7] Method details are in the STAR Methods. Because we studied bees during a time of relative natural food dearth, colony foraging focused upon this feeder and hornet attacks significantly reduced colony level waggle dancing as compared with when colony foragers were not attacked (0.1-fold, $F_{1,32} = 138.69$, $p < 0.0001$), increased the number of stop signals produced in the colony (15.5-fold, $F_{1,32} = 198.14$, $p < 0.0001$), and decreased the number of foragers visiting the feeder (0.3-fold, $F_{1,32} = 11.74$, $p < 0.0001$; Figure 1)

Foragers produced no stop signals when they were not attacked but gave a mean of 111 stop signals per bee within 10 min after being attacked ($F_{1,29} = 5{,}154.32$, $p < 0.0001$; Figure 2A). Attacked bees abandoned the dangerous feeder and spent >600 s inside the hive in contrast to the 143 s spent, on average, in the hive between feeder visits when they were not attacked ($F_{1,29} = 713.20$, $p < 0.0001$; Figure 2B).

Attacks significantly reduced the number of waggle dance circuits ($F_{2,58} = 949.97$, $p < 0.0001$; Figure 2C), which are positively correlated with a bee's perception of food quality and the number of nestmates recruited. In the control phase (no attacks and no stop signals received) foragers produced an average of 33 waggle circuits per hive visit, but this decreased to 5.3 waggle circuits per hive visit when they received stop signals (3.2 ± 2.4 stop signals per dance performance, mean ± 1 standard deviation), a 10.3-fold decrease in dancing. When attacked by hornets, foragers completely ceased waggle dancing (all pairwise differences significantly different, Tukey's HSD test, $p < 0.05$).

Bee behavior type was significantly correlated with dopamine levels in bee heads, as measured by high performance liquid chromatography-electrochemical detection ($F_{3,94} = 60.66$, $p < 0.0001$; Figure 3B). Waggle dancers (collected after five dance circuits) had significantly higher dopamine levels than all other bee behavior types, including control bees (foragers that remained in the hive, Tukey's HSD test, $p < 0.05$). Stop signalers are foragers that were attacked by hornets at the feeder and produced stop signals in the hive and, as predicted, had significantly lower dopamine levels (Tukey's HSD test, $p < 0.05$). Head dopamine levels decreased in waggle dancers to levels found in control bees, even if waggle dancers simply received stop signals and did not have any direct experience with predators (received stop signals behavior type, Tukey's HSD test, $p < 0.05$; Figure 3). Huang et al.[2] showed that brain dopamine decreased when dancers completed their dances. However, stop signals usually do not immediately terminate dances, and thus dancers that

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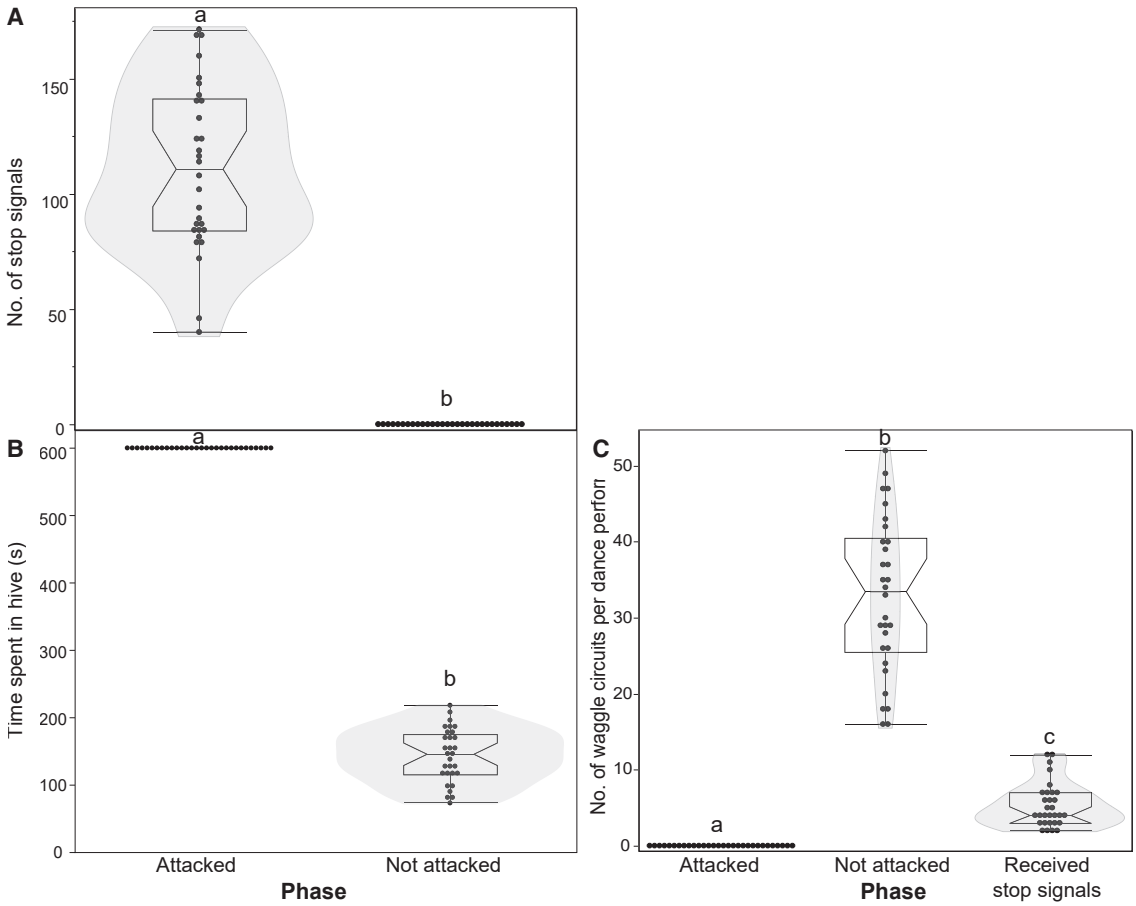

Figure 2. Hornet attacks increased stop signaling and decreased waggle dancing by individuals

Hornet attacks (A) increased stop signaling by attacked bees (n = 30 bees), (B) increased the time spent inside the hive (n = 30 bees), and (C) decreased the number of waggle dance circuits per dance performance (n = 30 bees). Waggle dancers that received stop signals performed fewer dance circuits. Different letters indicate significant differences (F tests, $p < 0.0001$; for the number of waggle circuits, Tukey's HSD test, $p < 0.05$).

received stop signals were still dancing and were also collected after producing five dance circuits, identical to waggle dancers that never received stop signals.

The large changes in total dopamine that we measured (Figure 3) include dopamine that has been released and dopamine that remains stored within synaptic vesicles. The relative proportions of such free and stored dopamine are unknown for the honeybee brain, but Huang et al.[2] likewise found major changes in similar amounts of brain dopamine: an average decrease of 72% after approximately 1 min of feeding or a decrease of 69% after approximately 1 min of waggle dancing. Likewise, Nouvian et al.[22] reported that levels of central brain dopamine levels increased by 22% after 3 min in bees that were exposed to bee alarm pheromone and that also responded aggressively by extending their stingers. Our use of the brain with some additional head tissue instead of only brain tissue is a limitation of our study, but our results support research demonstrating the role of increased brain dopamine in waggle dancing.[2]

Multiple studies have shown that orally feeding honeybee workers with biogenic amine-laced sucrose solutions, such as octopamine[23] or dopamine,[24,25] is an effective way to increase brain levels of a specific biogenic amine and to influence behavior. We fed bees sucrose solution with dopamine and significantly increased their head dopamine levels ($F_{1,14} = 8.69$, $p = 0.01$) by 1.2-fold and reduced the aversive effects of hornet attacks (Figure 3C). In detail, bees were allowed to visit a sucrose solution feeder, with or without 100 μg/mL dopamine, for approximately 30 min and were then attacked with hornets after the bees landed and imbibed sucrose for 30 s. Bees consuming the dopamine sucrose solution spent 6.9-fold more time on the feeder after an attack than bees that fed on the control sucrose solution after an attack ($F_{1,56} = 1,117.05$, $p < 0.0001$). Upon returning to the hive (a trip that took approximately 30 s), bees that consumed dopamine produced fewer stop signals (0.03-fold as compared with control bees, $F_{1,56} = 70.79$, $p < 0.0001$), more waggle dance circuits ($F_{1,56} = 8.50$, $p = 0.005$), and spent less time inside the hive before returning to the feeder (0.3-fold, $F_{1,56} = 81.55$, $p < 0.0001$) as compared with controls. By all these measures, dopamine consumption reduced the aversion elicited by hornet attacks.

We thus provide the first evidence that receiving a signal associated with negative food conditions (the stop signal) is sufficient to decrease brain dopamine levels in waggle dancers, even when these dancers have not experienced peril. Individuals

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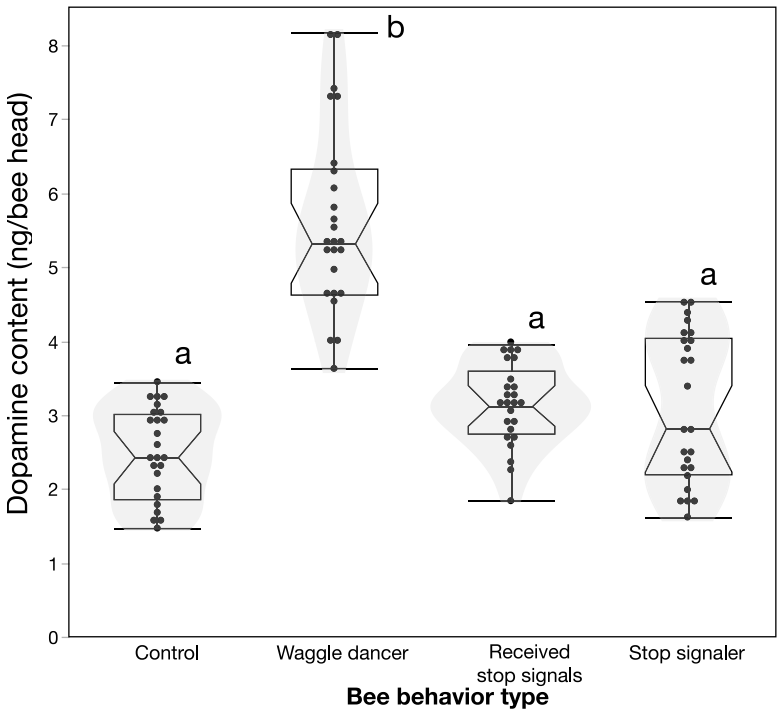
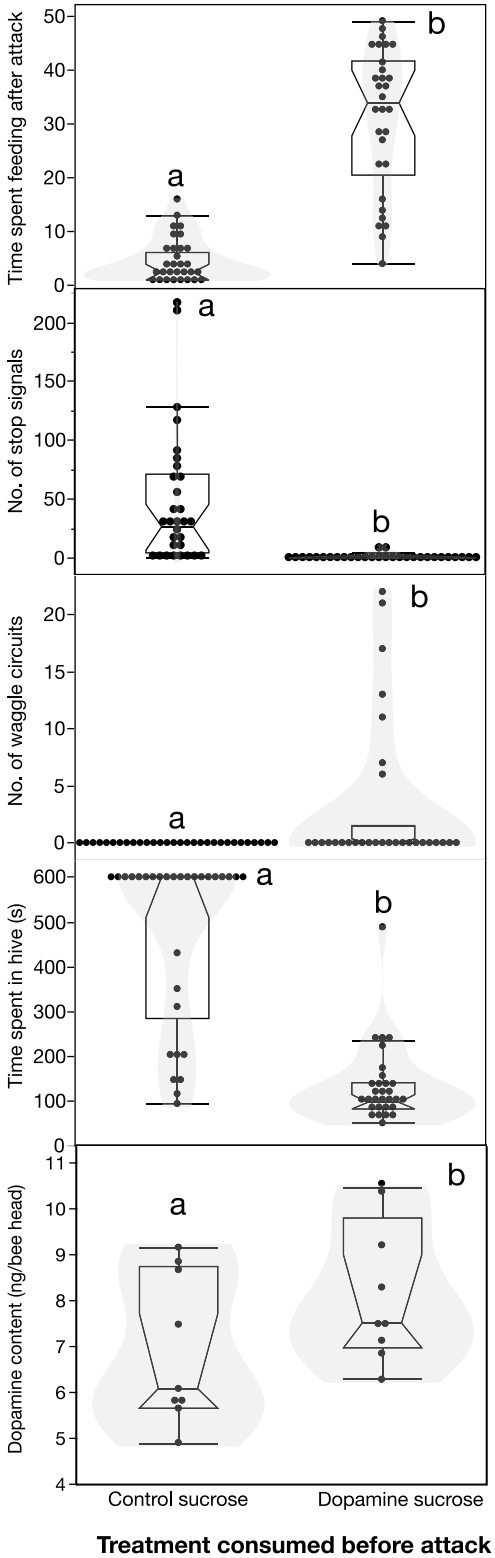
A *Vespa mandarinia* attacking a honey bee at a feeder

B Treatment effects on dopamine



C Effects of increasing dopamine



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produce fewer waggle dance circuits if their direct experience of food indicates lower quality.[26] Stop signals also cause receivers to produce fewer waggle dance circuits. Stop signals may therefore reduce dance perception of food quality by decreasing the level of food wanting. Given that colony needs have a major influence on honeybee behavior and wanting, including defensiveness and multiple types of resource gathering, this dopaminergic wanting system is likely shaped by multiple emergent aspects of the colony as a superorganism.

As predicted, increasing bee dopamine levels reduced the aversiveness of hornet attacks. Attacked bees that fed on the dopamine sucrose solution spent significantly more time feeding after being attacked, produced fewer stop signals when they returned to the hive, and waggle danced more than bees that were also attacked but fed on pure sucrose solution. Attacked bees fed dopamine also waggle danced significantly more than control bees. Thus, the fear-inducing effects of an attack can be countered by pharmacologically increasing bee dopamine levels. A natural stressor—attacks by the predatory hornet, *V. mandarinia*—can therefore reduce brain dopamine levels and cause foragers to pass-on such stressful information via stop signals that also reduce brain dopamine levels in recipients. Artificially increasing dopamine levels can reverse these effects.

When bees had pharmacologically elevated dopamine levels but were attacked by a hornet, these foragers, by design, always survived. The surviving bees could therefore be viewed as "winners" in an aggressive contest, and these bees behaved more like the winners of an attack than losers: they did not retreat to spend extended time inside the nest, produced fewer stop signals, and spent more time feeding after being attacked. Elevated dopamine is also associated with more fighting and aggression in honeybee queens,[27] and queen-like workers that won fights had higher brain dopamine levels than losers.[28] Nouvian et al.[22] reported that pharmacologically increasing brain dopamine levels led to increased aggression. The increased foraging behavior of our dopamine-fed bees, despite being attacked, also agrees with other studies. Higher levels of honeybee brain dopamine are associated with foraging,[2,5] and increased flight activity,[29] and pharmacologically elevating brain dopamine levels increased the foraging of harvester ants.[30]

Dopaminergic neurons are found in different brain regions in flies[31] and bees,[32] and serve multiple functions, including regulating movement, pleasure, motivation, arousal, and memory.[33] In honeybees, dopamine may serve two different roles: acting as a gain control system to down-regulate neural responsiveness and, separately, facilitating the learning of aversive stimuli.[32] Compartmentalized increases in dopamine may explain how dopamine can specifically affect forager perception of the hedonic value of food. Given these diverse functions, the large overall increases in dopamine that we and others[2] have measured with respect to foraging motivation are somewhat surprising. Dopamine biosynthesis and degradation may potentially play a role. The hydroxylation of L-Tyr to L-DOPA by tyrosine hydroxylase is a rate-limiting step in insect dopamine biosynthesis[33] and has rapid kinetics.[34] Similarly, insect dopamine is rapidly degraded via oxidation by tyrosinase (phenoloxidase)[35] and laccase.[36] Studies on the kinetics of these enzymes in the honeybee brain could yield further insights. In addition, detailed studies of changes in free and vesicular dopamine in different dopaminergic neural clusters are needed, and the signaling cascade that begins with the receipt of the vibratory stop signal and leads to brain dopamine reduction should be elucidated.

In mammals, both dopamine and octopamine are linked to the hedonic value of food.[37] There may be a similar, modular reward-processing system in *Drosophila* in which a sweet taste can initiate a octopamine signal that strengthens memory via specific subsets of dopamine neurons targeting the mushroom bodies, which are crucial for learning and memory.[38] Octopamine is involved in appetitive reinforcement in *Drosophila*,[39] and similarly, octopamine signaling through honeybee VUMmx1 neurons can substitute for sugar reinforcement.[40] Octopamine release is also essential for memory recall following honeybee memory consolidation.[41] Future research on the role of these biogenic amines in honeybee wanting could therefore reveal new parallels between the functions of octopamine and dopamine in mammals and insects.

STAR★METHODS

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

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- QUANTIFICATION AND STATISTICAL ANALYSIS

Figure 3. Attacks by hornets and stop signals reduced bee dopamine levels, but increasing dopamine reduced the aversive effects of attacks

(A) The image shows foragers at a feeder being attacked by a *V. mandarinia* hornet.

(B) The effect of treatment on bee head dopamine levels (measured per bee head) is shown. Control bees are foragers that used to visit the feeder, but had not visited for >1 h (n = 25 bees). Waggle dancers are foragers that continued to visit and dance for the feeder (n = 25 bees). Bees that received stop signals continued visiting the feeder and were never attacked but received stop signals (n = 25 bees). Stop signalers are foragers that continued to visit the feeder and performed stop signals back inside the hive after being attacked by a hornet (n = 25 bees).

(C) The effects of feeding bees with dopamine or control sucrose solutions for 30 min before attacks (n = 60 bees for behaviors, n = 18 bees for dopamine levels). Different letters indicate significant differences (Tukey's HSD test, $p < 0.05$).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.03.072.

A video abstract is available at https://doi.org/10.1016/j.cub.2023.03.072#mmc1.

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AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization and design of this research. S.D., G.G., T.L., Z.W., and J.L. conducted the experiments. J.C.N. analyzed the data. S.D., K.T., and J.C.N. contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium 1-octanesulfonate	Macklin	S817852-25g
EDTA	BioFroxx	1340GR500g
Citric Acid	Chemical Reagent	500g
NaH_2PO_4	Macklin	S817780-500g
Acetonitrile	Mreda	M157-4L
ddWater	N/A	N/A
$Na_2S_2O_5$	Macklin	S818096-100g
EDTA	BioFroxx	1340GR500g
$HClO_4$	Chemical Reagent	500ml
Deposited data		
All numerical data used in this paper	The authors of this paper	https://doi.org/10.5281/zenodo.7758140
Other		
Video camera	Sony	Model HDR-PJ790
Dopamine Hydrochloride	J&K	907334-5g

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shihao Dong (dongshihao@xtbg.ac.cn).

Materials availability

The materials used were purchased from standard chemical suppliers, who are listed in the materials and equipment table. No custom materials were created or used.

Data and code availability

- Standardized Excel data have been deposited at Zenodo and are publicly available as of the date of publication. The DOI for this data is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Honey bees (*Apis mellifera*)

We used three *A. mellifera* colonies housed in observation hives kept in the Southwest Biodiversity Laboratory in Kunming, China from June to September 2022. All the colonies were healthy, based upon standard apiary inspection practices, and each contained approximately 5,000 bees as determined by Liebefelder photographic estimation.[42] Each colony contained one queen and a mixture of male and female bees in the ratio typical for *A. mellifera* colonies. The observation hives (55.4x17x64 cm) each contained two combs (43.5x23 cm per comb): one frame of brood and one frame of honey and pollen. The hive was connected by a 2.2 cm inner diameter and 25 cm long tube through the wall to the outside. Upon entering the hive, all bees were shunted by a wood and beeswax divider to one side of the lower comb, the dance floor, where all waggle dances occurred.[43]

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METHOD DETAILS

Data collection

To record waggle dances and stop signals, a monitor placed a microphone (Movo LV1 Lavalier Microphone) on a thin metal rod connected to a Sony HDR-PJ790 camera and recorded waggle dances and stop signals as previously described.[8]

Experiment 1: Effect of predator threat on waggle dance and stop signal

We trained 30 bees from each colony to feeders containing a 50% sucrose solution (w/v) and located 120 m from the focal colony by gently capturing departing foragers at the hive entrance in a vial and releasing them slowly at the training feeder.[8] Bees from different colonies were trained to different directions to avoid competition for the same food source. The feeder consisted of a 70 ml vial (8 cm high) inverted over a circular plastic disk with 18 feeding grooves through which the sucrose could flow. After being filled with sucrose solution, the vial was inverted on the plastic disk and placed over a blue paper circle. Over 30 foragers could visit this feeder without crowding. When they were feeding, we gently and individually marked them with different paint (Edding 750) colors on their thoraces and verified that they came from the focal colony. As needed, we removed bees with an aspirator to prevent feeder overcrowding. After a few visits, the bees began to waggle dance for the feeder.

After bees had been trained to a feeder for 30 min, we recorded the colony-level response to hornet attacks on foragers at the feeder. We then counted the total number of foragers, waggle dancers, and stop signals for 10 min before hornets were presented at the feeder. In this ''before'' phase, the feeder monitor took a clean wood rod (1 m long) without a hornet attached and swept its tip 2 cm above the feeder, while recording the number of foragers visiting the feeder. Meanwhile, the hive monitor recorded the total number of waggle circuits and stop signals each 10 min with a microphone (see above) and a Sony HDR-PJ790 camera on the dance floor, where most waggle dances and stop signals occur. To scan for stop signals, the monitor moved the microphone approximately 1 cm away from bees on the comb and in a zigzag pattern, up and down at a speed of approximately 0.2 m/s over the surface of the dance floor.[8]

To generate hornet attacks without injuring foragers, we tethered a hornet (*V. mandarinia*) to the end of the rod with a stiff wire wrapped between the hornet's thorax and abdomen that allowed the hornet to flap its wings, as it does during natural attacks. We placed the hornet 2 cm away from the feeder, but without contacting bees. At this distance, bees exhibited alarm and usually fled. The feeder monitor swayed the wood rod at a speed of approximately 0.2 m/s while keeping the hornet 2 cm away from the feeder and recording the number of bee forager visits per 10 min. We conducted six replicate tests per colony, making only one test per day with any given colony between 14:00-17:00. All tests were conducted on sunny days.

We also measured the responses of individual foragers before and after hornet attacks. In the control phase before attacks, we swept a clean rod without a hornet 2 cm away from the individually marked foragers. When the focal forager departed the feeder, the feeder monitor informed the hive monitor via a two-way radio to prepare for the forager's arrival and record its waggle dancing, stop signaling, and hive visit duration. We also recorded these behaviors for the same focal forager after it was attacked by a hornet (see above). In total, we recorded 10 bees per colony with three colonies. Once a forager returned to the hive after an attack attempt, it produced the most stop signals in the first 10 min and then decreased stop signal production. We thus based our 10 min observation periods upon this natural decay in the rate of individual stop signaling. In these individual observations, we also recorded the effects of stop signals on waggle dancers that received stop signals (3.2±2.4 stop signals per dance performance, mean±1 standard deviation) by counting the number of waggle dance circuits performed during a hive visit (defined as beginning when a forager returned to the hive and ending when she left the hive). Foragers typically remained inside the nest for an average of 143 s before returning to the feeder when they were not attacked. When they were attacked, they usually did not return to the feeder for the rest of the day. We therefore chose 10 min as the observation interval.

Unlike the whole-colony observations, which counted stop signals produced on the colony dance floor, the individual observations counted stop signals produced by individually tracked foragers. In the control no-hornet attack phase, we recorded an average of 7.4 stop signals per 10 min scan from the colony-level measurements, but we counted 0 stop signals per 10 min when tracking individuals. This difference in the number of stop signals produced in the no-hornet attack phase likely arose from the larger number of bees being sampled in the whole-colony observations. In both whole-colony and individual measurements, stop signaling sharply increased when bees were attacked by hornets.

Experiment 2: Head dopamine levels of foragers after different predation and signaling treatments

We caught the four bee behavior types once they returned inside the hive (control bees, waggle dancers, stop signalers, and bees that received stop signals) to quantify the dopamine levels in their heads. Control bees had visited the feeder multiple times, but stopped visiting and remained in the hive for more than 1 h at the time of capture (n=25 bees). Waggle dancers continued to visit the feeder and began dancing immediately upon their return to the hive. We caught these waggle dancers after they had performed five waggle dance circuits (n=25 bees). Stop signalers are foragers that continued to visit the feeder and were attacked by a tethered *V. mandarinia* hornet after they had begun collecting nectar for 30 s. Upon returning to their hive, these attacked foragers began to perform stop signals (n=25 bees). Receivers of stop signals were foragers that were waggle dancing for the same feeder but had not experienced any hornet attacks or hornet presence on the feeder. We likewise captured these stop signal receivers after they had completed five waggle dance circuits (n=25 bees).

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During attacks, the feeder monitor recorded the identity of all bees visiting the feeder to ensure that the waggle dancers from which we measured head dopamine had never experienced the predator at the feeder. We also replaced the feeder with a clean one after attacks to remove any odors associated with predation: potential honey bee alarm pheromone or hornet odors.

To preserve head dopamine levels upon capture, we immersed the bee immediately into liquid nitrogen. All samples were then held in a -80 °C freezer until processing. To process each head, we used a clean scalpel to remove the antennas, eyes, mandibles, and the proboscis and cuticle from each bee head. To the remaining tissue, we added 500 μL of protein precipitation solution (0.4 mol/L perchloric diluent, 2.6 mmol $Na_2S_2O_5$, and 2.7 mmol EDTA) into a 1.5 ml microcentrifuge tube. This method differs from that of Huang et al.,[2] who dissected out the bee brain and measured dopamine levels in the brain alone.[2] We then ground this head remain with an electric grinder (Tiangen) at 8000 rpm. After thorough grinding for 1 min, we added another 500 μL of protein precipitation solution, vortexed the tube, and centrifuged it at 4 °C and 13,000 rpm (15,871 RCF, Eppendorf Centrifuge 5424R) for 30 min. The supernatant was then filtered through a 0.22 μm membrane and transferred into 1.5 ml micro vials for HPLC-ECD analysis (HPLC: Waters 1525; ECD: Waters 2489). We used a chromatographic system consisting of a solvent delivery pump (Waters 1525 binary HPLC pump, Singapore), an autosampler (Waters 2707), a C_{18} reversed-phase column (inner diameter 4.6 × 250 mm, average particle size 5 μm) maintained at 39 °C, and a UV detector (UV/visible detector, Waters 2489, Singapore). The detector was set to 264 nm, and its signals were recorded and processed with Breeze 2 software. The mobile phase contained 100 ml of acetonitrile, 1.7 mmol of sodium 1-octane sulfonate, 64 mmol of anhydrous sodium dihydrogen phosphate, and 50 μmol EDTA, which was adjusted to a pH of 3.0 with citric acid. We then used a 0.22 μm pore diameter filter membrane to remove impurities. Finally, we removed the bubbles with an ultrasonic instrument (SB-800D, Scientz, ultrasonic frequency of 40 kHz) for 30 min. During the detection, the flow rate of the mobile phase was kept constant at 1.0 ml/min. We used dopamine standards to quantify the content of dopamine in the bee head. We injected a control and three concentrations of dopamine standards (0, 6, 60, and 600 ng/ml) before each run and then calculated the amount of dopamine amounts in each sample by comparing the peak areas between the sample and the standards.[2]

Experiment 3: Effect of consuming dopamine on forager behaviors after hornet attacks

To test if consuming dopamine in sugar solution and thereby elevating dopamine levels would alter the behavior of bees after hornet attacks, we trained individually paint-marked bees to feeders as in Exp 1. However, in Exp 3, bees from the same colony were trained to two feeders that were each 120 m from the focal colony and spaced 3 m apart. During training, the feeder contained pure 50% w/v sucrose solution. At the start of each trial, we replaced the training feeders with a control feeder containing pure 50% w/v sucrose solution and an experimental feeder containing 50% w/v sucrose solution with 100 μg/ ml dopamine hydrochloride (Cas No. 62-31-7, J&K Scientific, China) based upon the methods of Harris et al.[44] Multiple studies have shown that orally feeding honey bee workers with dopamine-laced sucrose solutions is an effective way to increase bee brain dopamine levels and to influence behavior.[24,25]

After bees had repeatedly visited the feeder for approximately 30 min over multiple visits, we randomly selected foragers at the feeder in a visit and attacked them with a tethered *V. mandarinia* hornet after bees had imbibed the nectar for 30 s. We then recorded how long they remained foraging at the feeder after these attacks. When the attacked bees departed, we used two-way radios to inform the hive monitor of the attacked forager's departure back to the hive where the monitor recorded the number of waggle dance circuits that it made upon returning, the number of stop signals that it produced, and measured the total time that it spent inside the hive.

To estimate the rate of dopamine consumed by foragers, we compared the body mass of foragers before and after feeding on the dopamine sugar solution. We trained 10 bees from each colony (three colonies, a total of 30 bees) to the dopamine-laced experimental feeder and then immediately captured them and used brief CO_2 anesthesia to induce bee immobility. We then weighed these bees with an analytical balance (model ES1205A, Shanghai, China, accuracy of 0.01 mg). Once the bees had recovered from the anesthesia, they were released and allowed to forage over multiple trips at the experimental feeder until they showed no foraging hesitation. On their next trip to this feeder, we allowed them to collect dopamine sugar solution for 30 s before capturing them, CO_2 anesthetizing them, and weighing them again. We then calculated the amount of dopamine consumed from the mass of sucrose solution consumed.

Bees imbibed 93.3±23.3 ng dopamine/bee/s (mean±1 standard deviation) at the experimental feeder. There was no significant difference in the consumption of the dopamine solution by individual foragers from the three colonies (Analysis of Variance, ANOVA, $F_{2,27}$=1.41, P=0.26, with colony as a fixed effect). Not all of the dopamine imbibed would have been absorbed by the foragers, and thus we also measured the dopamine in bee heads after feeding on the dopamine hydrochloride sucrose solution by freezing a randomly selected subset of bees (three replicates per colony resulting in six bees per colony from three colonies, half of which fed on dopamine sucrose solution) in liquid nitrogen after 30 s of feeding (after approximately 30 min of total feeding over multiple visits) and then following the procedures in Exp. 2 for dopamine quantification.

QUANTIFICATION AND STATISTICAL ANALYSIS

We used JMP Pro 16.1.0 statistical software. For the colony-level data (Exp 1), we used Univariate Repeated Measures Models with the colony as the repeated measure and experimental phase as a fixed effect. We log-transformed the number of stop signals and feeder visits per 10 min.

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To examine the effects of treatment on individual production of stop signals, waggle dance circuits, and the amount of time they remained inside the hive (Exp 1), we ran Repeated Measures Mixed Models (REML algorithm) with the colony as a random effect, individual bee identity nested within the colony (a random effect), and experimental phase as a fixed effect. The number of stop signals, waggle dance circuits, and hive visit duration were log-transformed based upon inspection of model residuals.

To determine the effect of bee type on bee head dopamine levels (Exp. 2), we ran a Mixed Model (REML algorithm) with colony as a random effect and bee type as a fixed effect. To test for the effects of feeding bee dopamine (Exp. 3), we also ran a Mixed Model (REML algorithm) with colony as a random effect and treatment type (control sucrose solution vs. sucrose solution with dopamine) as a fixed effect. For these Exp. 3 models, we log-transformed time on feeder, time inside the hive, the number of stop signals produced, and the number of waggle circuits performed. To determine the effect of feeding on dopamine on bee head dopamine levels, we ran a Mixed Model (REML algorithm) with colony as a random effect and bee type (feeding on control sucrose solution with no dopamine or experimental sucrose solution with dopamine) as a fixed effect (Exp. 2).