

# Non-associative learning underlies pollination interaction of pollinators and flowering plants

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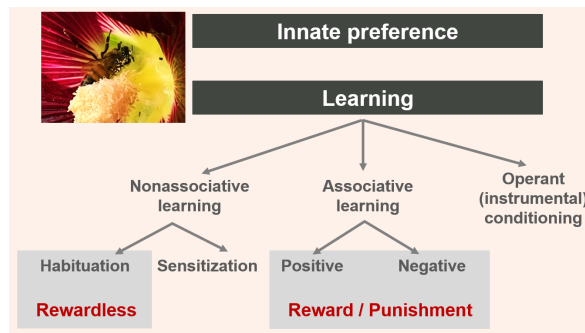
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**Abstract:** The behavioral response of pollinators is significantly influenced by the prior experience of flower visiting. Learning of pollinators, including non-associative learning, associative learning, and operant conditioning, is determined by the presence or absence of rewards during the flower visiting experience. Here, we indicate that process of non-rewarding flower (empty flower) visiting coincident well with the behavioral paradigm of non-associative learning. Habituation, one of non-associative learning, most likely modulates the pollinating behavior patterns of empty flower visitation. Moreover, we propose that the process of habituation recovery, including spontaneous recovery and dishabituation, may also modulate the behavior of pollinators, which leads to ecological consequences of long-distance pollen dispersal and high outcross pollination rate. We believe that utilizing the methodology of non-associative learning behavioral neurobiology paradigm to investigate pollinator behavior will establish novel insights into the sensory responses and neural activity of pollination behavior in the pollination systems.

**Keywords:** pollinating behavior, cognition, non-rewarding flowers, habituation, long-distance dispersion of pollen, high outcrossing rate

## INTRODUCTION

Pollination is a key ecosystem function for the maintenance of both wild plant communities and agricultural productivity [1]. In addition, cross-pollination enriches the genetic variability of plant populations and increases the potential for climate change adaptation [2,3]. Close to 90% of flowering plant species require pollinators for reproduction [2], and in turn many animal pollinators rely on floral resource for multiple types of rewards [4]. The prerequisite of pollination interactions between animal pollinators and flowering plants is the occurrence of asymmetrical signal communication between them, with the flowers as signal sources and pollinators as floral signal receivers. Pollinators, especially pollinating insects, employ multisensory mechanisms, such as olfactory and visual systems, to perceive and interpret plant signals [5,6]. These signals are subsequently processed by a vast network of neural circuits, which ultimately produce the appropriate floral visitation response [7]. Hence, understanding the pollinators' behavioral and neural principles constitutes a



**Figure 1** Distinct learning paradigm with their corresponding pollination scenarios. Learning can be broadly categorized into non-associative learning (habituation and sensitization), associative learning and operant (instrumental) conditioning. Associative learning occurs through the pairing of two previously unrelated stimuli, whereas non-associative learning occurs in response to a single stimulus. Associative learning occurs in rewarding or punitive flower visits. Non-associative learning comprises sensitization and habituation, while habituation is more likely to occur in rewardless visiting behavior.

crucial aspect to comprehend the plant-pollinator interaction.

The overwhelming majority of pollinators comprise wild insect species, predominantly from the orders Diptera, Lepidoptera, and Hymenoptera [8,9]. Over 70% of known angiosperms are pollinated by insects [10]. Moreover, pollinators are widely considered to play a crucial role in the origin of angiosperms, and their long-term mutual interactions have driven the biodiversity of both plants and insects [10–12]. Within these principal pollinating taxa – encompassing bees, moths, and flies – extensive knowledge has been amassed regarding the intricacies of insect behavioral cognition mechanisms [13–16]. The behavioral response of insect pollinators to a signal is regulated by a combination of two cognitive processes: one is the preexisting innate preferences of the insect, and the other is derived from the learning of the acquired experience [14,17,18]. Learning enables pollinators to establish correlations among environmental stimuli, and thereby anticipate the likelihood of future resources or threats [19]. Different forms of learning, including non-associative learning (habituation and sensitization), associative learning and operant (instrumental) conditioning, are determined by the presence or absence of rewards during the learning process [20–22] (Figure 1). Pollinators visit flowers in order to obtain rewards [23]. In the rewarding pollination systems, associative learning is considered a suitable cognitive process [18]. However, a considerable number of plants in nature usually do not provide any rewards to pollinators, which are called “empty flowers” [24–27]. The cognitive and neural mechanisms that underlie the learning in empty flower visitation remain inadequately elucidated. In this article, we will explain the learning process of empty flower visit in terms of a form of learning, the non-associative learning, and describe its behavioral characteristics, possible molecular and neural principles governing pollination decision, and potential ecological implications in the context of empty flower pollination.

## LEARNING IN INSECT POLLINATION BEHAVIOR

Pollinating insects have flexible and complex learning abilities, while in most pollination systems, learning determines the adaptive response of pollinators to different floral signals [28–30]. Insects exhibit innate preferences for certain signals, and these innate behavioral responses to such signals are heritable, and

considered to be hardwired in the neural circuit [31–34]. Compared to innate preferences, learning exhibits acquired plasticity and is defined as an update in the response to external signals, enabling the learner to alter their innate behavioral responses to a particular signal [35,36]. This process is modulated by a variety of mechanisms at different neural circuit sites [37–39]. Within the context of pollination activities, the external signals encompass the entire suite of floral traits experienced during pollinating process that stimulates the insect's sensory system. This includes, but is not limited to, the colour, shape, and scent of flowers [6,40,41]. The prior experienced signals influence the visiting choices of pollinators, resulting in either positive or negative behavioral responses in subsequent interactions with the signals presented by plants [22].

Nectar, pollen, or other structures produced by plants can be provided to pollinators as rewards during the pollinator visiting process, in which insect pollinators learn the association between floral signal stimuli and rewards. This leads to an improvement in the response of insect pollinators to the cues from flowers of the same species, thereby improving the attractiveness of flower signals to pollinators during the next flower visits [42–44]. At the same time, in several investigations, pollinators also have exhibited learning behavior towards non-rewarding flowers, as evidenced by their avoidance behavior towards empty flowers they have previously encountered [45–47]. Among the three distinct forms of learning, the significance of associative learning in flower-visiting behavior has been extensively highlighted in a considerable body of research literature. In contrast, non-associative learning has been relatively neglected in the field of pollination biology. In the following text, we will describe in detail and compare these forms of learning and their corresponding pollination scenarios, including non-associative learning, associative learning, and operant (instrumental) conditioning.

### **Non-associative learning**

Non-associative learning is the oldest and most fundamental form of learning and refers to becoming less (habituation) or more (sensitization) sensitive to a sensory signal through exposure to signals [48,49]. In non-associative learning, an organism exhibits a variation in its behavior response as a result of exposure to successive repeated stimulus to a signal, independent of any reinforcement (reward or punishment) that may be associated with the stimulus [21]. Sensitization occurs as a result of exposure to a strong or meaningful stimulus, such as a noxious stimulus or a highly desirable one like a potential mate. Habituation arises from the repeated occurrence of a specific stimulus without any consequence, leading to a diminished behavioral response to the stimulus [49,50]. The habituation has significant adaptive value as it enables animals to avoid unnecessary energy expenditure, preserving energy reserves for responses to valuable stimuli [51]. While both habituation and sensitization are forms of non-associative learning, they are triggered by different types of stimuli and have opposite effects on an organism's response to those stimuli.

When pollinators visit unrewarding flowers, habituation may be involved in this process, as the stimulation by the flower signal is not accompanied by the consequences of obtaining a reward [37]. Studying habituation can help us better understand the behavior and perception mechanisms of pollinators under empty flower visitation. From the perspective of neural mechanisms, habituation occurs when sensory neural activity is inhibited under conditions of repetition or continuous stimulation [52,53]. From the ecological perspective, however, the general ecological implication of habituation is to reduce the response towards temporarily less ecologically significant signals and to highlight key signals in the environment [22,49,52].

Therefore, the key to the habituation of pollinators lies in the habituation of sensory systems that recognize floral signals, including olfactory and visual systems [50,52]. During empty flower visits, pollinators may recognize flower signals as temporarily unimportant ecological signals and prompt habituation to those non-rewarding flower signals.

Habituation, as well as sensitization, as forms of non-associative learning are now recognized to occur across a wide range of organisms, from bacteria to vertebrate [36]. Behavioral characteristics of habituation have been reported in various pollinating insect groups. For instance, a behavioral study in bumblebees (*Bombus impatiens*) demonstrated the habituation to visual signals. When choosing in a radial arm maze, bumblebees exhibited innate preferences for artificial blue and yellow signals, but the proportion of choices for these preferred signals significantly decreased after two training sessions. This preference was restored upon the introduction of new visual stimuli [54]. Additionally, in honeybees, the sensitization and habituation of appetitive responses to sucrose, measured through the proboscis extension reflex (PER), are often used to assess the level of non-associative learning in bees [55,56].

Given that the occurrence of flower-visiting behavior is a recognition of signals for pollinators, it essentially represents the pollinator's needs for the benefits required for survival [23,32]. Therefore, flower-visiting behavior has similarities to the behavior of insects in gaining benefits from interactions with non-plant organisms. These interactions beyond pollinator-plant interactions can provide new insights into the occurrence of non-associative learning during pollination. In the research of interactions between insect predator and prey, or the relationship between the host and parasite, have provided detailed reports of non-associative learning and habituation phenomena in insect behavior. For example, the parasitoid wasp *Trisolcus basalus* showed progressively shorter searching times for the next 48 h when repeatedly exposed to the traces of its host without an oviposition reward [49]. Another study on host and parasite showed that the diminishing responses of parasitoids to chemical indicators of host presence (*Nezara viridula* chemical contact footprints) are achieved by habituation [57]. In these examples, insects were stimulated by signals without receiving food or other forms of reward, which is consistent with the experience of pollinators in empty flower-visiting. Based on the extensive research on non-associative learning and habituation in insect behavior, we hypothesize that habituation may be prevalent in the flower-visiting behavior of pollinators, modulating their behaviors during repeated non-rewarding floral visits. This habituation is likely involved in the reduction of pollinators' responses to non-rewarding plant signals [42,58]. The habituation of cognitive processes, as described, may provide the underlying basis for pollinator interactions with empty flower plants.

### **Associative learning**

The associative learning allows pollinators to learn to associate a neutral stimulus (the “conditioned stimulus,” or CS), which does not originally elicit a behavioral response, with a stimulus of biological significance (the “unconditioned stimulus,” or US), which elicits an innate, often reflexive, response. After the two stimuli are associated, the previously neutral conditioned stimulus can enable animals to prepare for these biologically significant events [59]. Signals from the same flower are multisensory and diverse, and neutral stimuli are part of these flower signals for which pollinators have no innate preference [23,49]. Pollinators evaluate the amounts of nectar or pollen rewards when visiting rewarding plants, and associate the

rewards with the neutral floral signal stimulus under the process of associative learning. Reward-related associative learning is thought to increase the visitation frequency of pollinators or decrease the searching and processing time of the flowers, leading to an improvement in the foraging efficiency of those pollinators [14,22,60].

The earliest study of learning and memory in pollinators was done with honeybees and examined the olfactory conditioning of the proboscis extension response (PER) [15,59]. Sucrose was most commonly used as an unconditioned stimulus during associative learning training in PER, and the corresponding changes in PER responses to conditioned stimuli occurred after training [15]. Associative learning not only occurs in social pollinating insects such as bees, but also plays an important role in the pollination behavior of many important solitary and semi-social pollinators of plants, such as wasps, flies, and adult Lepidopterans. For example, bumble bees learn to choose among artificial flowers of different colours after they have been extensively trained [40]. The butterfly *Byasa alcinous* showed both reward and nonreward learning abilities in whilst foraging among artificial flowers of different colours [45].

In the context of the behavioral paradigm of associative learning, the US can be either rewarding or punishment [22,61]. In the classical associative learning theory, negative and positive associative learning are the two important components, among which positive associative learning leads to attractive responses to the conditioned training signals, and negative associative learning leads to aversive responses [62]. Negative associative learning can also lead to a decrease in pollinator preference for flower signals, but it is important to note that negative associative learning needs to be distinguished from non-associative learning. Negative learning of animals occurs on the premise that the signal needs to be associated with a strong negative unconditioned stimulus called punishment (such as electric shock), which usually does not occur in empty flower pollination (Figure 1) [61,63]. There are some exceptions, such as the flowers of trigger plant (*Stylosidium*) that strike pollinators forcefully, sometimes even knocking them off the flowers, which possibly leads to negative associative learning [64]. Negative associative learning may lead to insects acquiring memories of moving away from the signal more quickly. Besides, it is difficult to employ the classical associative learning theory and paradigm to study pollinating behaviors in the non-rewarding pollination systems, since there is no unconditioned stimulus (rewards) can be associated with a neutral floral signal stimulus in empty flowers. In general, non-associative learning refers to the reduction of behavioral response to a signal that originally elicits a response in pollinators after multiple unrewarding experiences. In contrast, negative associative learning may lead to aversive behavior towards neutral stimuli when they are associated with punishment. These two types of learning may be applied in different pollination scenarios (Figure 1).

### **Operant (instrumental) conditioning**

One special form of associative learning is operant conditioning. In operant conditioning, the animal's stereotyped behavior produces the "reinforcer", which is the stimuli or event that increases the likelihood or frequency of a particular behavior [65]. For example, rats have been shown to be trained to press a lever to obtain a food pellet reinforcer [66]. Whereas classical conditioning can be thought of as "learning to recognize", operant conditioning is "learning to do": an animal learns and optimizes a novel behavior based on feedback that results from its behavioral experiments [67]. Operant conditioning allows insects to produce relatively stereotyped behaviors which are called learned motor programs [67]. For example, Abramson

*et al.* [68] tested operant conditioning in bees using the cap-pushing response (CPR), which requires bees to exhibit behavior of pushing the cap to discover food sources. Generally speaking, operant conditioning is more commonly involved in studying the artificial reinforcement of specific behaviors in pollinating insects, and is rarely involved in studies of natural pollination behavior [66,69].

## **BEHAVIORAL FEATURES AND MECHANISMS OF NON-ASSOCIATIVE LEARNING AND HABITUATION IN POLLINATION**

Habituation, as a part of non-associative learning, has been elucidated for its behavioral characteristics and neural mechanisms in several insect species. Especially in the field of pest control, insect habituation to attractants or deterrents represents a significant focal point in the efficacy of pest trapping. However, the impact of non-associative learning on pollinator behavioral patterns is currently lack of attention.

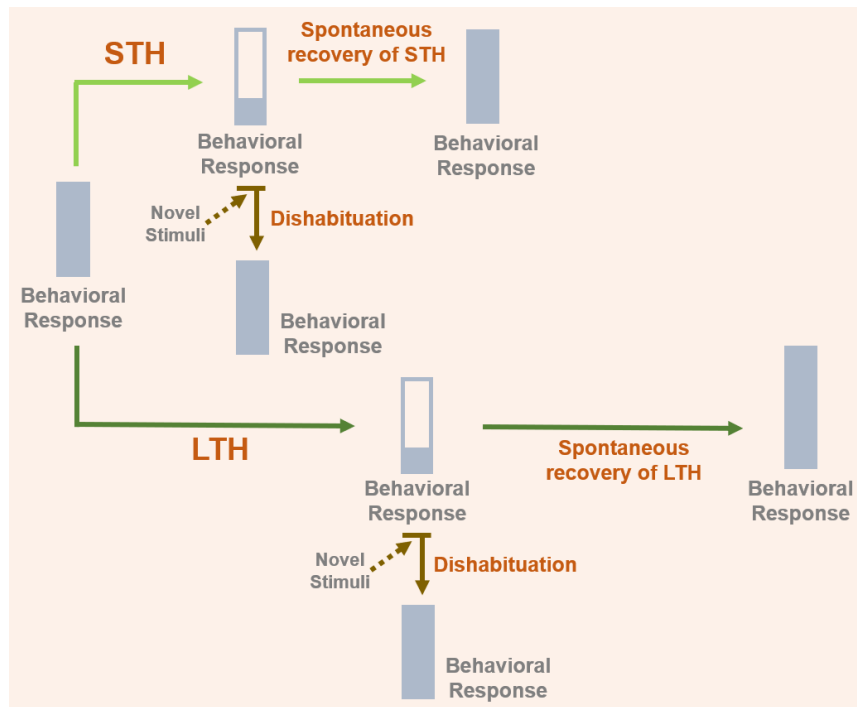
### **The features of habituation**

Generally, the habituation of animals has a behavior feature of a decreased response to frequent or continuous signals [70,71]. This reduction is usually a negative exponential function of the number of stimulus presentations [71,72]. Besides, if the stimulus is withheld, the response tends to recover over time (spontaneous recovery) [73]. The property of spontaneous recovery can be distinct from the “fatigue and/or sensory adaptation” recovery produced by the sensory system that leads to faster response decay as the stimulus intensities increase [57,73–75].

When animals are exposed to multiple stimuli signals, the habituation is stimulus-specific [50,51]. The perception of external signals in animals involves multiple sensory systems, while habituation in one sensory system does not affect the reception of signals by other sensory systems, such as olfactory habituation not influencing visual or tactile responses [51,72]. Furthermore, the specificity is reflected in the reception of different signals by the same sensory system. For instance, in *Manduca sexta* larvae, gustatory habituation to salicin affects the perception of caffeine, which shares the same neural signaling pathway, but does not impact responses to other compounds, including aristolochic acid or *Grindelia* extract [76]. Based on this characteristic, we speculate that the pollinator’s habituation of certain plant signals will not affect its visitation to other plants. These features of habituation can serve as a framework for investigating the neural mechanisms and behavioral patterns of empty flower-visiting behavior.

### **Reversibility of habituation-induced behavior**

Habituation has the important property of its reversibility, which can be summarized as spontaneous recovery and dishabituation (Figure 2). Taking olfactory habituation as an example, the spontaneous recovery of insects to olfactory stimuli has been extensively studied, and two different timescales have been distinguished. In an olfactory avoidance experiment, *D. melanogaster* habituated to ethyl butyrate (EB) showed short-term habituation (STH) after 30 min of exposure and long-term habituation (LTH) after 4 h of exposure [77]. STH can be recovered spontaneously with a half value period of 30 min and can be recovered by

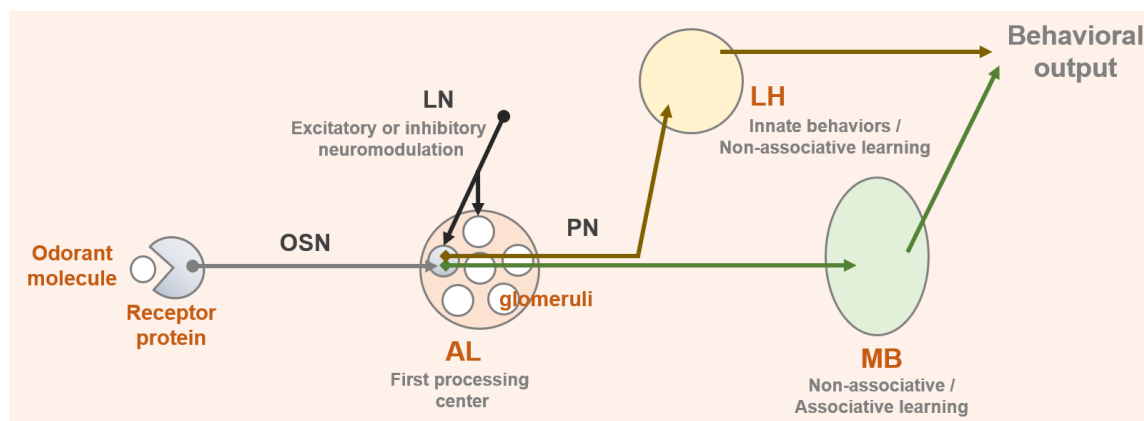


**Figure 2** Diagram illustrating the behavioral characteristics of habituation and its reversibility. The height of the gray rectangles in the diagram represents the degree of a pollinator’s behavioral response preference to a certain signal. The horizontal dimension in the diagram suggests the different time scales at which habituation occurs.

mechanical stimulation or yeast odor. Besides, LTH is elicited by 4-day exposure to either odorant, lasts up to 6 days, and shows odorant selectivity [77,78]. In the investigations of pollinator visiting behaviors, the duration of flower visitation by pollinators typically falls within the time range of short-term habituation (STH), especially for those non-rewarding flowers, with the average visitation time for empty flowers being shorter than that for rewarding flowers [79]. However, for plants that grow in clustered communities, pollinators are exposed to a wide range of signal environments during floral foraging, which increases the possibility of LTH occurring under such conditions. This property may correspond to the important behavioral feature of empty flower pollination, that is, the pollinator’s avoidance of non-rewarding signals usually cannot be maintained for a long time. For example, the pollinating wasps showed 4- and 60-min duration of short-term avoidance of the deceptive odor signals [80]. Generally, the spontaneous recovery of habituation could occur under two different conditions. In one condition, when the stimulus is withheld, the behavioral response of insects tends to undergo a time-dependent or frequency-dependent spontaneous recovery to this stimulus [71]. The duration and frequency of the signal received by insects lead to STH or LTH, resulting in different durations of habituation recovery [50,77].

In another condition, presentation of a novel (usually strong) stimulus results in recovery of the habituated response, which is called dishabituation [71]. In addition, strong stimulation of another sensory system may lead to the dishabituation of the original sensory system [37]. For example, *Drosophila* flies habituated to the odor ethyl butyrate (EB) quickly recover their behavioral preference for EB when stimulated by yeast odor [50,77]. The “time window” for behavioral valence recovery was different in the scenarios of spontaneous recovery and dishabituation. In the scenario of pollination ecology, when pollinators visit nonrewarding





**Figure 3** Diagram of the neural system's response and processing of external signals (using the olfactory neural system of *Drosophila* as an example). Black text represents names of neuron, while brown uppercase letters represent structures in the *Drosophila* brain. OSN, olfactory sensory neuron; LN, local neuron; PN, projection neuron; AL, antennal lobe; LH, lateral horn; MB, mushroom body.

flowers, the reversibility of habituation could mediate the response of pollinators to the flower signals, allowing pollinators to recover their preference for the signals after pollinators depart from the flower for a period of time. It has been reported that the innate preferences of pollinators such as butterflies, bumblebees, and hawkmoths can be replaced by experienced behavioral responses; however, they are able to revert to their innate preferences when presented with novel stimuli [26].

### The neural and molecular mechanisms of insect habituation

The molecular and neural mechanisms of habituation have been detailed described in *Drosophila* and several invertebrates. For example, in the olfactory system of *Drosophila*, the architecture and layers of odor information processing, as well as the potential mechanisms of habituation, have been well elucidated [50,81,82]. In the peripheral olfactory system (antennae), odorant molecules bind to specific receptor proteins located on the membranes of olfactory receptor neurons (ORNs). These signals are then assigned to the first processing center, known as the antennal lobe (AL) in insects. Within the AL, neurons in specific olfactory glomeruli form synapses with projection neurons (PNs) and project to central brain structures, including the mushroom body (MB) and lateral horn (LH) in the brain, for information integration and the output of behavioral response. Neurons in the antennal lobe are also connected to local neurons (LNs), participating in excitatory or inhibitory neuromodulation [81].

Non-associative learning and memory rely on multiple mechanisms of plasticity at multiple sites of the neuronal circuits [37] (Figure 3). The latest research elucidated that learning and memory, including non-associative learning in insects occur in the MBs regions of the brain, while the LHs are involved in guiding innate behaviors mediated by olfaction [50]. Habituation is accompanied by enhancement of inhibitory synapses in primary sensory processing centers [52,77]. Besides, several observations strongly indicate that STH and LTH of olfactory avoidance response arise from central neurons, regulated by LNs, rather than peripheral neuronal changes [77]. The plasticity in the central neural circuit provides a basis for the formation and reversibility of habituation. Different neural mechanisms are involved in STH and LTH to signals in insects. STH, which occurs within seconds to minutes of signal exposure, depends on excitatory and in-



hibitory neural transmission to the lateral horn. In contrast, long-term habituation, which occurs after prolonged odor exposure, is located within the antennal lobe, and requires local inhibitory interneuron activity [52]. Synaptic protein phosphorylation has been proposed as a putative mechanism underlying the phenomenon of spontaneous recovery of habituation, whereas dishabituation is not the disruption of habituation but an independent superimposed process of sensitization [50,71].

The molecular mechanisms underlying the formation of STH and LTH are different. It is currently believed that the formation of STH is based on the covalent modification of pre-existing proteins, whereas the formation of LTH depends on gene transcription, protein translation, and the growth of new synapses [83,84]. Several conserved genes closely related to memory and cognition have been identified in the habituation process. In *Drosophila*, studies using gene knockout mutants found that *rutabaga* and *dunce*, the cAMP signaling pathway genes that were most frequently studied, were involved in behavioral plasticity and habituation in *Drosophila* and other mammalian species [73,85,86]. Furthermore, synaptic proteins, as a conserved family of synaptic vesicle-associated proteins, have been found to be associated with the formation of STH rather than LTH. After the LTH of odor signals, insects were observed to have an increase in the volume of olfactory glomeruli in the antennal lobe, possibly due to the growth of new synaptic. A key transcription regulatory factor, cAMP-responsive element binding protein (CREB), is involved in the transcription and translation control mechanism required for LTH but not for STH [50]. Besides, the molecular mechanism of dishabituation might be mediated by neuromodulators acting on the neural circuits responsible for the habituated response. Studies in *Drosophila* have found that neurotransmission is required via the neurons of MBs and from MBs to LH neurons, as silencing these neurons disables dishabituation [86]. Research in mice suggests that norepinephrine may be involved in this process [50], but in insects, this hypothesis is currently under investigation.

More important, the molecular and neural mechanisms of habituation are considered to be conserved in animals [37,87]. Habituation has been observed in organisms ranging from unicellular organisms such as amoebas and paramecia [71]. In animals with neural systems, research of the monosynaptic gill-withdrawal response in *Aplysia* and the startle reflex in *Drosophila*, the basic synaptic inhibition mechanism underlying habituation appears to be conserved [70,71,88,89]. For example, in the study of honeybees, habituation is achieved by enhancing the synaptic strength between local inhibitory neurons (LNs) and projection neurons (PNs), much like in *Drosophila* [52]. Therefore, we can apply the neural mechanisms and behavioral paradigms of non-associative learning in model organisms to study pollinator behavior in order to distinguish whether non-associative learning occurs in specific pollination behaviors.

## THE ECOLOGICAL CONSEQUENCES OF NON-ASSOCIATIVE LEARNING IN POLLINATION

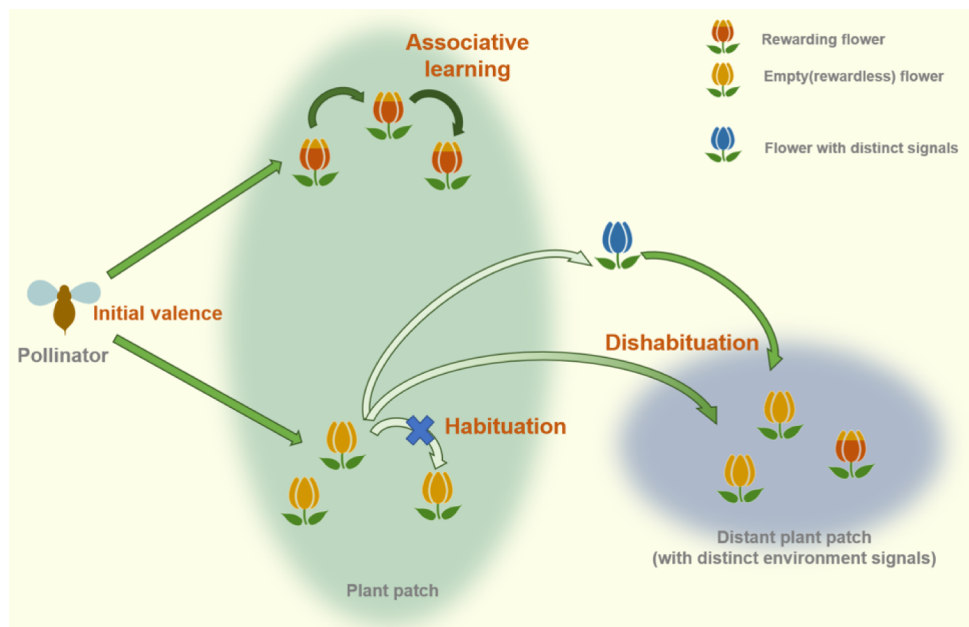
Associative learning makes pollinators more inclined to visit flowers of the same species continuously in a plant community, to increase intraspecific pollen transfer and reduce the deposition of interspecific pollen [22,90,91]. These abilities establish the constancy of flowers, that is, the pollinator's ability to focus on a single flower species during the foraging process [47]. However, in rewarding pollination systems, most pollinators' flight among flowers occurs within plant patches, rarely between plant patches [92]. In fact,

longer pollination distances are of great significance for plants. Pollination within the patch is more likely to lead to geitonogamy (one form of self-pollination) of plants, which may not only reduce the fitness of their offspring, but also lead to the waste of pollen [93]. If pollinators were able to move over long distances, gene flow between widely separated plant patches might persist by avoiding self-pollination among individuals of the same genotype [94]. Compared to rewarding pollination systems, flower foragers often respond to non-rewarding flowers by visiting fewer flowers per inflorescence or patch but moving further between inflorescences or patches [24].

In fact, unrewarding plants are more common than expected. There are different causes of empty flowers, including deceptive plant species that are genetically non-rewarding to pollinators, and rewarding plants that are depleted of nectar or other rewarding resources temporarily. Species with genetically stable rewardless flowers, known as deceptive pollination flowers, have evolved in at least 32 angiosperm families and especially in the Orchidaceae family [46,95–97]. Besides, by virtue of insufficient nectar secretion or drained by animal foragers, Thakar *et al.* [98] reported that the proportion of temporary empty flowers was as high as 68% in a community composed of 24 plant species under the circumstance of excluding pollinators. In those communities containing empty flowers, the characteristics of habituation may lead the pollinators to fly across different patches or communities, resulting in significant ecological consequences [99].

Previous studies have shown that in natural communities dominated by empty flowers, the flower visit behavior of pollinators and their consequences can be described at two levels. One is that pollinators quickly abandon repeated visits to similar flowers, resulting in a temporary decrease in flower-visiting rate [54,100–102]. For example, pollinators of *Ophrys sphegodes* quickly learned the locations and scents of the deceptive orchids and tended to avoid visiting those deceptive individuals within minutes [99]. The other is that pollinators will visit less frequently between flowers on the same plant or adjacent plant individuals, thus creating a long-distance dispersal of pollen and increasing the outcrossing rate of plants [99,103–105]. Some researchers have shown that the pollen dispersal distance of empty flowers is far greater than that of rewarding flowers, resulting in a density- and frequency-independent of pollen removal and seed setting rate [104,106,107]. In our opinions, the processes of habituation and dishabituation of pollinators may be involved in the modulation of their pollinating behavior during empty flower visits. Therefore, the long-distance dispersal of pollen and higher outcross pollination rates in the non-rewarding pollination systems could be considered as the ecological consequence of non-associative learning.

Here, we use the paradigm of non-associative learning to analyze the flower-visiting behavior patterns of pollinators. Pollinators have an initial behavioral response to the floral signals before the experience of visiting flowers, which may come from a combination of innate preference and acquired learning [108]. Following the visit to the empty flower, pollinators will undergo two successive pollination processes. In the first process, pollinators become insensitive to signals from nonrewarding flowers due to habituation. The consequence of habituation will decrease pollinator preference for flowers with the same signal over a period of time, and increase the possibility that the pollinator departs away from flowers with the same signal. The habituation-induced variation in pollinator behavior valence has a significant impact on the pollen dispersal pattern of the host plant. In the second process, pollinators' valence can be spontaneously recovered over time or over distance, leading pollinators to restore their valence to the same flower signals, thus increasing the probability of revisiting [103]. After habituation occurs, spontaneous recovery or dishabituation on different time scales can be changed into different moving distances of pollinators. Thus, the longer the



**Figure 4** Schematic representation of behavioral characteristics of associative learning, habituation, spontaneous recovery of habituation and dishabituation. The shade of arrow colour indicates the value of insect’s preference response to the plant signals, while darker colours represent more positive preference to the plant signals.

recovery time after the habituation of the pollinator is, the more likely it is that pollinators will carry out the long-distance pollen dispersal, which increases the outcross pollination of plants. Moreover, the process of dishabituation caused by strong stimulus of the new signals may alter the process of behavior response revisability for the floral signals after leaving the plant patch. These strong stimuli of new signals may come from background signals when pollinators move to new plant patches, or from flowers with different floral signals (Figure 4).

## PERSPECTIVES

In this article, we suppose that the cognitive process of non-associative learning may also widely exist in insect pollinating behaviors, leading to a change in the pollinators’ preference for flower signals, especially occurring in those non-rewarding pollination systems. The ability of pollinators to recover their behavior valence to flower signals after habituation may be the basal cognitive mechanism for the occurrence of long-distance pollination and higher cross-pollination rate during empty flower visits. We highlight the process of non-associative learning modulates the pollinating behavior patterns of empty flower visitation, and for the first time suggest that the habituation and dishabituation from non-associative learning can be used as a behavioral neurobiological paradigm to study pollinators’ neural basis of the visitation of empty flower. This suggests a scientific approach to understanding the biological phenomenon that is more common than expected. With the intensive exploration of the neural basis and genetic mechanisms underlying sensory evolution in non-model species, we entered a new era to decipher the peripheral sensory, coding, or neural connectivity of the floral signals underpinning the different pollinating behaviors. As the simplest form of

learning, non-associative learning can provide a simple and new paradigm to exploit the peripheral detection and primary process of signals in pollinators during the visitation to the empty flowers, which can facilitate the out-cross pollination in plants and enriches plant genetic diversity and the potential for climate change adaptation [109–111].

The empty flower-pollinator interaction provides a more simple tractable system to understand the process involved in the sensory integration of multiple streams of floral signals because there is no reward interference in this interaction. Therefore, we believe that the approach of the non-associative learning behavioral neurobiological paradigm will lay novel insights on the variations in sensory response and neural activities of pollinating behavior in the pollination systems. We advocate for more studies on: (1) adopt the behavioral and neurocognitive paradigm in the study of pollinator dynamic responses behaviors to flower signals, to confirm the existence as well as to reveal the neural bases of non-associative learning in empty flower visitation; (2) conduct more researches on pollinator foraging behavior patterns and ecological consequences under nonrewarding pollination from the perspective of pollinator cognitive processes in the laboratory but especially in the considerably larger scale environment in the fields; (3) design the experiments to test whether potential non-associative learning is involved in the decision-making process of pollinators during the selection of floral signals. Understanding the modulation of outcross pollination behaviors through non-associative learning could help us to manipulate the pollinators to increase the fruit or seed sets in some special commodity crops that have strictly outcross mating systems, such as pears and oil tea (*Camellia oleifera*) [112,113]. More importantly, the in-depth exploring the neural mechanisms and rationales of pollinator behavior plasticity and modulation, in turn, can contribute to exploiting the stability and maintenance of pollination service in natural and agricultural ecosystems, as well as to design pollination service in ecosystem restorations in an increasingly unstable world [3,114,115].

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## Author contributions

W.F.Z. and Y.B.L. conceived of the presented idea. W.F.Z. wrote the manuscript with support from Y.B.L. All authors discussed the opinions and contributed to the final manuscript.

## Conflict of interest

The authors declare no conflict of interest.

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