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BRIEF REPORT

Response-Specific Behavioral Plasticity in Habituation Triggered by Repeated Visual Looming Stimuli in Foraging Bumblebees (*Bombus terrestris*)

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Habituation and dishabituation are fundamental adaptive processes that govern how animals respond to repeated stimuli. Habituation is defined as a decline in response to irrelevant stimuli, and dishabituation reactivates this response upon qualitatively different stimulation. Here, we explored these processes in bumblebees (*Bombus terrestris*) by exposing freely foraging individuals to a repeated overhead looming stimulus, followed by a distinct vibration. We identified three defensive responses—flight, disturbance leg-lift response, and startle—and found that only flight probability showed robust habituation and dishabituation. Disturbance leg-lift response remained consistently frequent, whereas startle initially increased and later declined when flight was reinstated. Our findings demonstrate clear habituation and dishabituation of defensive responses in bumblebees within a novel free-flying testing paradigm, providing initial support for response-specific plasticity mechanisms. The results underscore the importance of differentiating among multiple defensive responses to better understand the mechanisms driving habituation and dishabituation, suggesting that bumblebee defense strategies are finely tuned across multiple stimulus–response pathways.

Keywords: defensive response, dishabituation, disturbance leg-lift response, flight, startle

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Habituation and dishabituation are two important adaptive phenomena related to repeated exposure to irrelevant stimuli. Habituation involves a gradual decrease in response, while dishabituation entails the recovery of a habituated response following the introduction of a novel stimulus (Thompson, 2009). Since the seminal studies by Kandel and colleagues (Castellucci & Kandel, 1974; Pinsker et al., 1970), habituation and dishabituation have been studied in many invertebrate species from Nematoda (e.g., C. H. Rankin & Broster, 1992), to Annelida (e.g., Reyes-Jiménez et al., 2020), Platyhelminthes (e.g., Nicolas et al., 2008), and Arthropoda (e.g., Dissegna et al., 2020; Tomsic et al., 1993). Both of these learning processes are critical for the survival of animals, as they enable energy conservation by reducing unnecessary responses to irrelevant stimuli (habituation), while also allowing for the reinstatement of previously habituated responses when environmental conditions change and the stimulus may once again signal potential threat (dishabituation; Dissegna et al., 2018; Steiner & Barry, 2011). However, basic questions about the underlying habituation and dishabituation remain unanswered. For example, it is unclear whether the reduction in responsivity during the habituation process occurs at a response-specific level, selectively modulating only the behaviors directly elicited by the habituating stimulus, while leaving others unaffected.

To investigate this possibility, it is necessary to examine multiple behaviors simultaneously, which in turn requires an experimental approach that allows different responses to be expressed and measured in parallel. Paradigms that test freely moving animals are particularly suited to this aim, as they preserve the natural repertoire of behaviors that would be restricted in restrained preparations. For example, a study by McDiarmid et al. (2018) examined multiple responses to repeated optogenetic activation of nociceptor neurons in freely behaving *Caernohabditis elegans*. In line with response-specific habituation, they found that repeated noxious stimulation led to habituation of the duration and latency of a reflexive reversal response, an innate defensive reflex; however, the probability of responding to the stimulation remained unaltered, and the forward movements even accelerated. Therefore, although animals reduced the extent of their defensive reactions, they continued to respond to the repetitive stimulus, indicating that they had not disengaged attention from it. The authors suggested that this selective modulation of behavior may

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be adaptive: by minimizing energetically costly reflexive responses while maintaining sensitivity to the stimulus, the worms might be optimizing their chances of escaping away from the threat (McDiarmid et al., 2018, 2019).

However, noxious stimuli such as those used in the previous study are unlikely to be fully disregarded by animals, even after extensive habituation training. Moreover, habituation is defined as a response decrement to irrelevant stimuli (C. Rankin et al., 2009), with few studies reporting habituation to biologically relevant stimuli (Chiandetti & Turatto, 2019; Daniel et al., 2019; Lloyd et al., 2014). As a consequence, the response-specific habituation observed in McDiarmid et al. (2018) might reflect a specialized process—tied to the noxious nature of the stimulus—rather than a more general mechanistic characteristics of habituation.

Here, we studied response-specific habituation in bumblebees (*Bombus terrestris*). Bumblebees are an increasingly important model in behavioral ecology and comparative psychology (Chittka, 2017); however, only a few experiments have focused on their habituation (Plowright et al., 2006; Simonds & Plowright, 2004; Varnon et al., 2021), and none of them demonstrated dishabituation, so far. The majority of studies on bees' habituation have been conducted on *Apis mellifera* and have focused on appetitive behaviors of restrained bees, such as the proboscis extension reflex (Baracchi et al., 2017; Braun & Bicker, 1992; Scheiner, 2004). These works showed that habituation can occur for both unconditioned and conditioned stimuli, generally supporting the view that repeated sensory input reduces responsiveness. In addition, they provided evidence for several key characteristics of habituation described in other species, including spontaneous recovery after rest intervals, state-specificity, long-term habituation across repeated sessions, and lateralization (Baracchi et al., 2017, 2018; Bicker & Hëhnlein, 1994; Braun & Bicker, 1992; Haupt & Klemt, 2005; Scheiner, 2004). Studies on freely flying bees similarly reported habituation of visual pattern preferences and spontaneous recovery (Plowright et al., 2006; Simonds & Plowright, 2004), reinforcing the idea that these fundamental properties of habituation are conserved across paradigms and experimental conditions.

Innate defensive responses of bees and their modulation by experience have received less attention (but see Jernigan et al., 2018; Varnon et al., 2021). The habituation of the disturbance leg-lift response (Varnon et al., 2021) of bumblebees is one exception. In this response, a bumblebee lifts one or both middle legs as a warning signal that it is prepared to sting. The behavior is elicited by the proximal contact between the animal and an external object or by approaching visual stimuli. In Varnon et al. (2021), bumblebees (*Bombus impatiens*) were restrained in clear capsules and stimulated by repeatedly lowering the experimenter's hand above the apparatus and rotating it several times. In addition, after 10 repetitions of the sequence of movements, bumblebees were dishabituated by tapping on the capsule floor before being tested again with the original stimulus. Results show that disturbance leg-lift response proportion decreased across repetitions of the hand movement and increased in response to tapping, in line with habituation and the stimulus-specificity of habituation (i.e., the fact that a habituated response does not generalize to new stimuli; C. Rankin et al., 2009). However, after tapping, the disturbance leg-lift response returned to the habituation level, providing no evidence in support of dishabituation.

In our experiment, we tested the simultaneous habituation and dishabituation of different defensive responses of bumblebees, as in Ardiel et al. (2017). Contrary to Varnon et al.'s (2021), we chose to study their defensive behavior while they foraged on artificial flowers by simulating a potential predator approach, rather than restraining them. This more naturalistic setup allowed us to observe a broader range of defensive responses, such as flights away from the flowers, which closely resemble their typical behavior when threats occur as they forage. We developed a new habituation–dishabituation paradigm using a standardized visual looming stimulus to compare habituation and dishabituation across different responses to test how specific defensive responses are selectively modulated by habituation.

Materials and Method

Animals and Housing

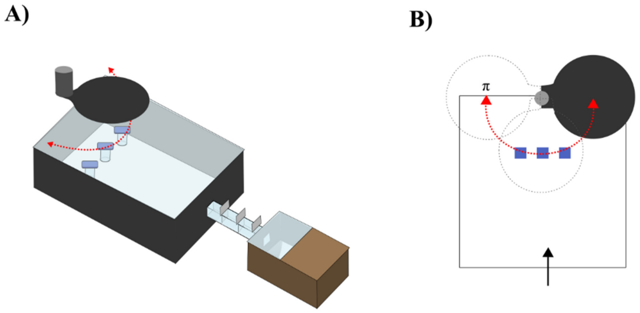
The sample comprised 37 bumblebees (*B. terrestris*) from three different colonies raised from Biobest (Westerlo, Belgium) standard hives. Colonies were housed in bipartite wooden nest boxes measuring 30 cm × 15 cm with a height of 15 cm. One section contained the nest, covered with plywood. At the opposite end was a 35 ml cylindrical feeder (74.5 mm × 31 mm) that dispensed 30% sucrose solution ad libitum. The floor of this section was covered with a layer of cat litter (Catsan Hygiene Plus, Mars Inc., Franklin, TN, United States) to absorb waste and debris. Each colony was provided with 7 g of pollen (Koppert, Berkel en Rodenrijs, The Netherlands) every 2 days, and the laboratory was maintained at a temperature of 23°C.

Apparatus and Stimuli

The nest box was connected to a rectangular testing arena (30 cm × 40 cm × 11 cm, length, width, and height) by a 30-cm-long clear plastic tunnel (Figure 1A). Access to the arena was controlled by three gates positioned along the tunnel. The floor of the arena and its lateral walls were opaque, while the arena roof was made of

Figure 1

Sketches of the Experimental Apparatus and the Looming Stimulus



Note. (A) Bumblebees accessed the arena from their nest box to forage from three blue square flowers. To reach the arena, they walked through a clear tunnel with three gates. Occasionally, a paddle controlled by the experimenter moved over their head while they foraged from the flowers. (B) The black paddle used as the habituation stimulus moved from 0 to π radians, paused for 1 s, and then rotated back, mimicking a flying predator approaching the bumblebees as they foraged from the blue square flowers. The black arrow indicates the entrance to the arena (outer black outlined rectangle). Sketches not drawn to scale. See the online article for the color version of this figure.

transparent plexiglass. Three square, blue, artificial flowers were placed at the opposite end of the arena entrance, at a distance of 10 cm from the rear wall. Each flower (2 cm × 2 cm × 0.5 cm) contained five drops (5 μl each) of 20% sucrose solution on the top, four at each corner, and one at the center, to encourage surface exploration. Each artificial flower was attached to a servo motor controlled via a custom Arduino circuit.

A black circular paddle (15 cm diameter) located 1 cm above the arena roof served as the habituation stimulus (Figure 1B). This paddle was mounted on the arm of a servo motor that rotated from 0 to π radians, paused for 1 s, and then rotated back at a rate of approximately 1 rad s^{-1}. The paddle's movement was manually controlled by the experimenter through a custom Arduino script. A rapid horizontal vibration of flowers was used as the dishabituation stimulus. The vibration lasted for 3 s at a frequency of 5 cycles s^{-1} with a total angular displacement of $\pi/4$ radians.

Procedure

Two days before the experiment, foragers were identified and tagged by gluing colored numbered labels (Opalith Zeichenplättchen Leuchtfarben, Weinstadt-Endersbach, Germany) on their thorax. Because we planned to test animals repeatedly, we opted to include in our experiment only the most motivated animals based on the number of complete foraging bouts bumblebees made, that is, how many times they left the nest box, visited one or more artificial flowers in the arena to collect the sucrose solution, and then returned to the nest. Specifically, we tested bumblebees that completed at least 15 consecutive foraging bouts on the day before the experiment.

On the experimental day, bumblebees were tested for five consecutive foraging bouts. In each of the first four bouts, bumblebees were exposed to a habituation sequence comprising 10 presentations of a looming stimulus at random interstimulus intervals between 5 and 15 s to prevent rapid habituation because of temporal predictability (Davis, 1970). The presentation of the stimulus was delivered at the scheduled interstimulus interval, independently of whether the bumblebee was on the flower, with each interstimulus interval beginning immediately after the end of the preceding looming stimulus. The fifth bout was identical to the previous four for 15 bumblebees; for another group of 15 randomly selected bumblebees (the dishabituation group), the final habituation sequence began after the bee returned to the flower following the end of a dishabituator[1] consisting in a vibration (see the Apparatus and Stimuli section). Bumblebees absent from the flower during stimulation on more than three consecutive trials were excluded. This criterion led to the exclusion of seven individuals from the final data set. Bumblebees' behavior was recorded by a camera at 120 Hz frame-by-frame resolution. We classified bumblebees' responses to stimulation. Two aspects of the response were analyzed: the response probability (the number of each response type within a bout divided by the number of paddle movements occurring in each bout, i.e., 20 movements per bout, with each event consisting of a forward and backward movement of the paddle) and the response magnitude (the average duration of a given response within a bout, measured in milliseconds).[2]

We analyzed the two variables using a linear-mixed effects model. Models were fitted using the lmer function of the lmerTest package in the R programming environment (R Core Team, 2019), with the factor "Foraging bout" in the fixed effect structure and by-individual intercepts. As statistical inferential measures, we provided: (a) type III-like two-tailed *p* values for significance estimates of linear mixed-effects models' fixed effects and parameters adjusting for the *F* tests the denominator degrees of freedom with the Satterthwaite approximation and (b) estimates of effect size based on partial eta squared (η_p^2; for the interactions and main effects of the *F* tests), and Cohen's *d* (for the Welch's two sample *t* tests).

Results

Classification of Defensive Responses to Visual Looming

We collected data from 742 defensive responses elicited by the looming stimulus that we classified into three categories:

1. The flight response, which involves the bumblebee abruptly flying away from the flower, is an adaptive response to a perceived threat, before returning to resume foraging (Figure 2A). This response is characterized by a mean duration of 1,700 ms ($SD = 922$ ms). To quantify this behavior, we measured the time elapsed from the moment a bumblebee initiated flight (leaving the flower surface) until it landed again on one of the flowers in the arena. Globally, this response accounted for 22% of the total.
2. The disturbance leg-lift response described by Varnon et al. (2021; Figure 2B). To quantify the disturbance leg-lift response, we recorded the time from the moment the bumblebee lifted its leg(s) until the leg(s) were lowered back to their resting position. The mean duration of the response was 459 ms ($SD = 207$ ms). This was the most frequent response, representing 63% of the total responses.
3. The startle response, which is a rapid, reflexive movement in which the bumblebee recoils its body in reaction to a perceived threat (Figure 2C). To quantify the startle, we measured the time from the onset of the receding movement until the bumblebee returned to a stationary position. This behavior is characterized by short durations, with a mean length of 126 ms ($SD = 204$ ms). It accounted for 15% of total responses.

Habituation and Dishabituation of Response Probability

The proportions of responses to the looming stimulus across foraging bouts are depicted in Figure 3A. Means and standard deviations can be found in Table S1 in the online supplemental materials. Overall, bumblebees showed a decline in the proportion of defensive responses to the looming stimulus from the first block ($M = 0.66$, $SD = 0.47$) to the fourth block ($M = 0.53$,

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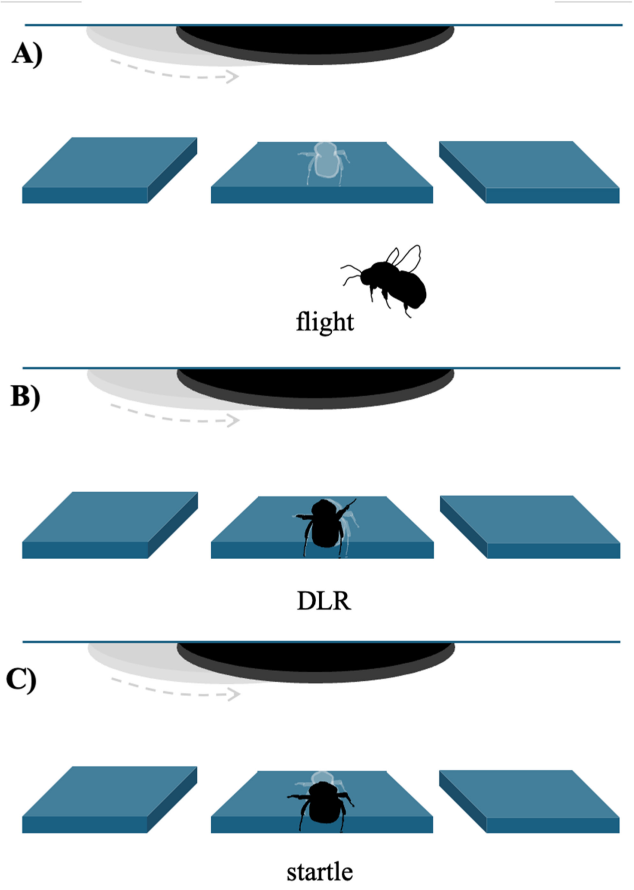
[1] As a preliminary analysis, we ran a correlation analysis to assess differences in the spontaneous recovery of the individuals' response duration depending on the differences in the interbout intervals (C. H. Rankin & Broster, 1992). The range of the interbout intervals was 16–37 min. The analysis revealed no significant effect on response duration during neither the habituation phase nor during the dishabituation phase (all *p*s > .050).

[2] We opted to average responses across trials within each bout to obtain a more reliable estimate of the animals' behavioral adjustment to repeated presentations of the looming stimulus. This decision was informed by preliminary analyses, which revealed a higher degree of trial-by-trial variability and the presence of potential outliers, whereas response measures aggregated at the bout level exhibited greater stability and consistency.

Figure 2
Sketches of the Bumblebees' Responses



Note. (A) The flight response: The bumblebee quickly flies away from the flower it was foraging from. (B) The DLR: The bumblebee lifts its middle legs in preparation for an attack. (C) The startle response: The bumblebee rapidly recoils its body in response to the disturbance. Semi-transparent white profiles indicate the starting position before the stimulus (upper black oval shape) appeared. Sketches are intended for illustrative purposes only. DLR = disturbance leg-lift response. See the online article for the color version of this figure.

SD = 0.49), $F(3, 81.29) = 2.99$, $p = .035$, $\eta_p^2 = .10$. Conversely, the proportion of null responses increased over the same period (first block: $M = 0.33$, $SD = 0.47$; fourth block: $M = 0.46$, $SD = 0.49$). Missing responses were a proportion of 0.01 of the total responses.

At a response-specific level, the proportion of flights decreased from foraging bouts 1–4, $F(3, 83.84) = 7.82$, $p < .001$, $\eta_p^2 = .22$. However, in the final foraging bout, the proportion of flights increased for the dishabituation group relative to the baseline habituation group, $t(23.52) = 1.82$, $p = .040$, $d = 0.75$. By contrast, the proportion of disturbance leg-lift responses remained stable across habituation foraging bouts, $F(3, 84.76) = 1.04$, $p = .376$, $\eta_p^2 = .04$, with no significant change in the final bout between the dishabituation and the habituation, $t(25.83) = 0.44$, $p = .329$, $d = 0.17$. Conversely, the proportion of startle responses progressively increased across foraging bouts 1–4, $F(3, 81) = 2.78$, $p = .046$, $\eta_p^2 = .093$, and decreased in the final bout in the dishabituation group compared to the habituation one, $t(25.50) = -1.92$, $p = .032$, $d = -0.76$.

Habituation and Dishabituation of Response Duration

Average response durations are depicted in Figure 3B. Means and *SD* can be found in Table S2 in the online supplemental materials. None of the response durations changed across foraging bouts 1–4, for flights: $F(3, 75) = 0.38$, $p = .765$, $\eta_p^2 = .02$; for disturbance leg-lift responses: $F(3, 76.23) = 1.02$, $p = .386$, $\eta_p^2 = .04$; and for startles: $F(3, 44.90) = 0.29$, $p = .826$, $\eta_p^2 = .02$. In the final bout, the dishabituation group increased the flight duration compared to the habituation one, $t(12.84) = 2.47$, $p = .013$, $d = 1.38$. The duration of disturbance leg-lift responses remained stable, $t(25.18) = 0.72$, $p = .237$, $d = 0.29$. By contrast, the duration of startles decreased in the dishabituation group, $t(14.76) = -2.04$, $p = .029$, $d = -1.06$.

Discussion

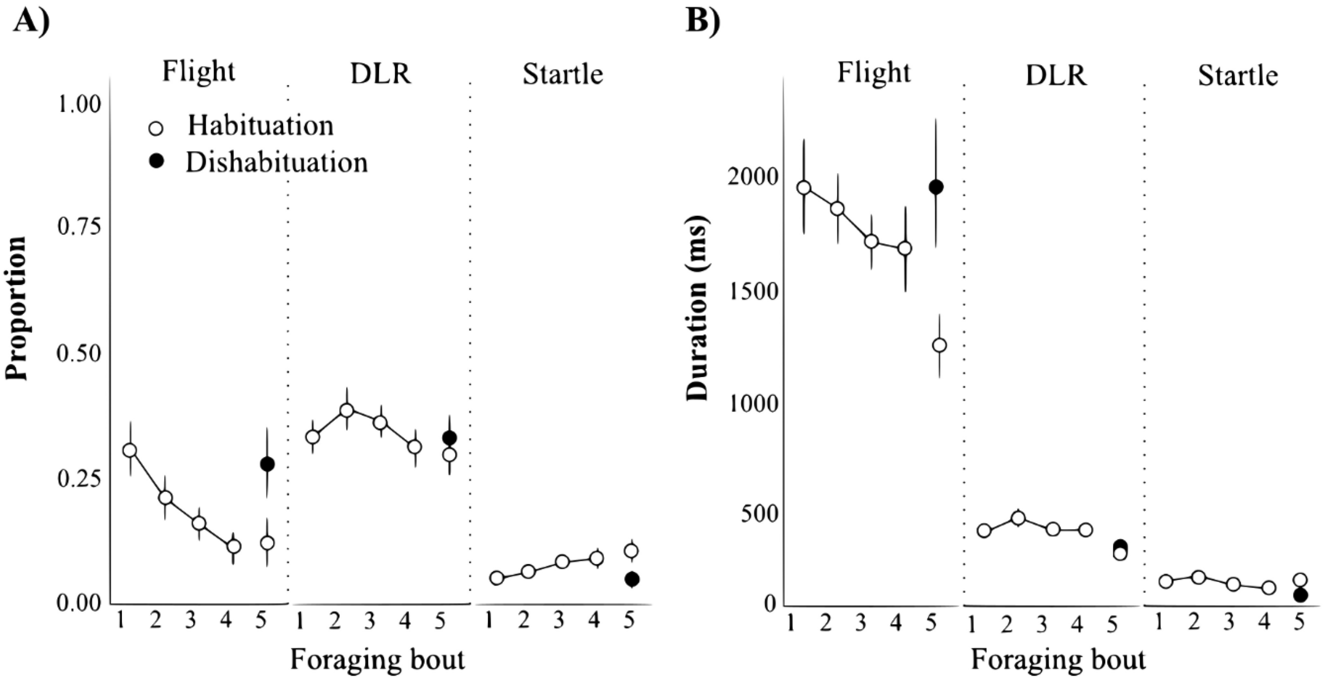
In this study, we examined how bumblebees adjust their defensive behavior in response to repeated presentations of an overhead visual looming stimulus, adopting a new free-flying habituation paradigm. We identified three defensive responses—flight, disturbance leg-lift response, and startle—elicited by visual looming stimuli in bumblebees and uncovered differences in how they undergo habituation and dishabituation.

In line with habituation, flight probability declined significantly with looming stimulus repetitions across foraging bouts and was reinstated (dishabituation) after a novel stimulus (i.e., vibration) was introduced, demonstrating that this response can be rapidly reverted when a new threat is introduced. By contrast, the disturbance leg-lift response probability remained stable across repeated exposures to the looming stimulus, denoting a lack of habituation under our experimental conditions. Finally, startle probability increased across the first four foraging bouts and decreased following the dishabituation stimulus.

Our findings are consistent with the hypothesis that habituation in bumblebees is response-specific, and that different components of the same response (initiation probability and duration) can be inhibited or reactivated independently. However, since the hierarchical organization of these three behaviors is still unknown, it is also possible that part or all of the defensive responses we examined may not operate in isolation, consistent with a shift between linked behaviors rather than independent response suppression. Specifically, the probability of startle responses initially increased following the novel vibration stimulus but subsequently declined, in contrast to the flight response pattern, which showed an exactly opposite pattern. One possible explanation for this apparent relationship is that startle responses and flight are hierarchically organized responses, with startle representing the initial stage of an "interrupted" flight response, in which bumblebees momentarily prepare to take off but do not fully commit to the action if the perceived threat does not escalate (Card, 2012). Future studies may adopt high-resolution kinematic analyses to better define the relationship between these potentially connected responses. A finer-grained kinematic analysis of responses in this paradigm may reveal modulations in the startle and disturbance leg-lift responses that were not detectable with our current measures. It is possible that these behaviors underwent subtle

Figure 3

Results of the Habituation and Dishabituation Test



Note. (A) Proportion of defensive responses as a function of the foraging bout. (B) Corresponding average durations. The fifth bout was identical to the previous four for 15 bumblebees (white disks); while another group of 15 bumblebees received the dishabituator before the final foraging bout (black disks, dishabituation). ANOVAs conducted on data from bouts 1–4 revealed no significant differences between the two groups (all *p*s > .05). Error bars represent *SEM*. DLR = disturbance leg-lift response; ANOVA = analysis of variance.

changes that remained obscured because they approached the floor of our measurement sensitivity.

The lack of habituation of the disturbance leg-lift response contrasts with previous findings by Varnon et al. (2021). The different result might be attributed to the different context in which the defensive response was elicited. Unlike our paradigm, where bumblebees were free to fly and forage, in the previous study, bumblebees were restrained in small capsules, preventing them from flight, and leaving disturbance leg-lift response as their primary available defense. In our paradigm, instead, bumblebees might have prioritized retaining this warning response (i.e., the disturbance leg-lift response) over more energetically costly responses (i.e., flight). Indeed, flight is not only expensive in terms of energy (Ellington, 1985) but also interrupts food collection. In contrast, disturbance leg-lift response offers a rapid, deterrent—a prelude to an attack or to a "fight" response—without requiring the abandonment of the sucrose reward. Therefore, context may be an important determinant of whether and how response-specific habituation occurs.

Another important finding is that response probability and response duration did not always follow the same trajectory under our habituation–dishabituation paradigm. Although flight and startle durations were generally stable during repeated exposures, their probabilities changed significantly. These dissociations indicate that initiation probability and magnitude (in terms of duration) of a response may be governed by partially independent processes (McDiarmid et al., 2018; Randlett et al., 2019). In other words, the bumblebees' initiation of defensive responses can be affected by learning processes in a way that is distinct from how long they sustain them once initiated. The present results extend previous observations indicating multiple, experience-dependent plasticity processes activated by habituation–dishabituation paradigms (McDiarmid et al., 2018). Given that in previous and in these studies response-specificity was observed in the context of defensive responses, this characteristics of habituation might be linked to the modularity of the defensive system (Bolles, 1970; Fanselow, 1994), and may serve an adaptive function by enabling a shift in defensive strategy—for example, minimizing energetically costly responses while preserving alertness to potential threats.

Our paradigm to test habituation–dishabituation in free-flying bees can be exploited by future studies to explore several unresolved questions regarding the dynamics of habituation and dishabituation under more natural, unrestrained conditions, thereby extending its utility as a model for studying experience-dependent plasticity. For example, future work could extend the number of within-bout trials to investigate patterns of within-bout habituation and recovery, or whether habituated responses show spontaneous recovery with faster rehabituation across bouts, or how interstimulus interval length influences habituation dynamics.

In general, our results support the hypothesis that habituation may occur along specific stimulus–response pathways as originally proposed by Groves and Thompson (1970), and reveal distinct experience-dependent modulations even within finer components of a single response, such as initiation probability and response duration. This fine-grained plasticity underscores the adaptive nature of habituation, allowing organisms to calibrate their behavioral responses with precision.

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