Smoke Conditions Affect the Release of the Venom Droplet Accompanying Sting Extension in Honey Bees (Hymenoptera: Apidae)

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Abstract

Honey bees (Apis mellifera) (Hymenoptera: Apidae) are social insects that have evolved a coordinated defensive response to ensure colony survival. Their nests may contain valuable resources such as pollen and nectar that are attractive to a range of insect and mammalian intruders and need protecting. With sufficient provocation, honey bees will mobilize and sting intruders, who are likely to incur additional stings. To inspect and manage their colonies, beekeepers apply smoke to decrease the likelihood of being stung. The use of smoke is a ubiquitous beekeeping practice, but the reasons behind its efficacy remain unknown. In this study, we examined the effects of smoke on honey bee defensive behavior by assessing individual sting extension responses under smoke conditions. We applied a brief voltage to the bee, ranging from a mild to a strong perturbation, and assessed four components of the sting extension reflex using two types of smoke. We found that smoke did not influence the probability of sting extension, but it did affect whether a venom droplet was released with the stinger. The venom droplet was more likely to be released at higher voltage levels, but this effect was significantly reduced under smoke conditions. Based on these results, we propose that the venom droplet coincides with greater agitation in individual bees; and smoke reduces the probability of its release. We speculate that the venom droplet serves to amplify the sting alarm pheromone, and smoke, in its ability to reduce droplet formation, may indicate that less alarm pheromone is released.

Key words: hops, lupulin, burlap, sting extension reflex, defensive behavior

Honey bees (Apis mellifera) are social insects that coordinate a suite of defensive behaviors to ensure colony survival. Their nests may contain valuable, energetically demanding resources in the form of pollen and nectar; while harboring brood, the reproductive queen, and the bees themselves. These resources need protecting from a range of intruders. These include robber bees, which are bees from other colonies typically with high pathogen loads that threaten resource availability and risk disease (Kuszewska and Woyciechowski 2014); or other insects such as wasps, moths, and beetles. Mammals, such as bears and skunks, also pose great destructive risk in search of honey and brood protein. To protect themselves, bees mount a colony-level defensive response which is one the most well-known (and feared) honey bee behaviors (reviewed in Nouvian et al. 2016).

Guard bees are usually present at the entrance of hives and though their numbers are few (only 10–15% of adult worker bees become guards, Moore et al. 1987), they represent the first line of defense. Guard bees patrol the entrance of the hive, and search for any creature or object approaching the colony (Arechavaleta-Velasco and Hunt 2003). Guards are very sensitive to vibrations, odors, color, and movement (Graham 1992), and can discriminate between nestmates and intruders (Breed et al. 1992). When a guard bee is disturbed, she will raise her abdomen and release alarm pheromone by opening her sting chamber and protruding her stinger. She also fans her wings, presumably to aid in the dispersal of her pheromone to alert the colony (Bortolotti et al. 2014). As pheromone is released, the number of bees at the entrance of the hive increases (Maschwitz 1964). Defender bees may rush out of the hive entrance running in circles or zigzags, or assume an aggressive posture (Free 1987, Bortolotti et al. 2014). This posture might include a slightly raised body with wings extended, antennae waving, and open mandibles (Ghent and Gary 1962, Free 1987). At this stage, bees are ready to mobilize at further provocation. Additional disturbance is likely to lead to the stinging of the intruder. Once stung, the chances of receiving additional stings become significantly increased (Free 1961, Ghent and Gary 1962).

To inspect and manage colonies, beekeepers use bee-smokers to direct smoke at the entrance and inside of their hives. Smoke reduces defensive behavior and allows the beekeeper to manipulate...
frames of bees without being stung. This practice is very effective, and used extensively by beekeepers around the world (Graham 1992). Despite being an integral beekeeping practice, reasons behind smoke effectiveness in reducing stings are not well understood. One of the most compelling arguments is that smoke disrupts chemical communication among bees. The alarm pheromone, for instance, may be masked by smoke such that its many compounds become indistinguishable in the odor plume. Or perhaps smoke weakens worker bee sensitivity to alarm pheromone by affecting olfactory transduction in the antennae sensilla. There is evidence in support of the latter. It was found that smoke reduced the electroantennograph response of honey bee antennae to both alarm pheromones and a floral odor, suggesting that smoke interferes temporarily with olfaction in general (Visscher et al. 1995). Apart from disrupting chemical communication, another argument is that smoke may function as a distraction. Smoke induces honey bees to ingest honey from storage cells, and because of this activity, they are less likely to sting (Free 1968, Newton 1968). Smoke may mask the intruder’s odor, or divert attention away toward the more immediate threat of a perceived fire in the area. Altogether, the defensive response is an example of collective action based on recruitment and amplification processes within the hive (Millor et al. 1999). If smoke affects one, or a combination of these processes, or other processes entirely, defensive behaviors are likely to be diminished.

We conducted a study to evaluate the effects of smoke on honey bee defensive response by assessing individual honey bee sting extension responses under smoke conditions. In this way, we could carefully monitor how perturbation with increasing voltage steps affects the sting extension reflex and whether this reflex is modified by smoke. There is evidence demonstrating that though there is some peripheral autonomy governing the sting extension reflex under voltage stimulation, there is considerable central control in this paradigm suggesting agitation in bees is being tested (Burrell and Smith 1994, Tedjakumala and Giurfa 2013). We chose two different sources of fuel to create two distinct smoke types; burlap—a common material used by beekeepers—and hop (Humulus lupulus) pellets comprised of dry hop cones from female plants. Lupulin, produced by the female flowers of hops, is a known sedative (Reilly 1906, Van Cleemput et al. 2009). We then applied a brief voltage to the bee, ranging from a mild to a strong perturbation and assessed the sting extension response under smoke and nonsmoke conditions. We hypothesized that smoke would reduce at least one of the four components of the sting extension reflex.

Methods

Bees

Bees were collected at the Carl Hayden Bee Research Center in Tucson, AZ, during August of 2016. Bees were taken from the entrances of European colonies, Apis mellifera ligustica, headed by queens from Pendell Apiaries (Stonyford, CA) in morning and afternoon sessions. These bees were presumed to be a mixture of guard and forager bees. Immediately prior to each session, 60–70 bees were collected in total from the entrances of three hives or more using soft forceps. They were kept briefly in a cage (less than 60 min) and individually transferred into scintillation vials (less than 10 min) in preparation for restraint and experimentation. Bees were randomly assigned to one of three conditions (-) No smoke, (+) Burlap smoke, and (+) Hops smoke. This process was repeated for 4 days to assess the effects of these conditions at varying levels of perturbation administered by an electric shock of 1, 2, 4, or 8 volts (V). Each condition tested the responses of 40 bees at each voltage. In total, 480 bees were used for the experiment.

Experimental Setup

Restraint

Prior to smoke application and testing, bees were chilled on ice until immobile. Once a bee was immobile, she was positioned between two brass plates outfitted with grooves supporting the segment between her neck and thorax, and thorax and abdomen (Fig. 1) similar to reports by Núñez et al. (1997) and Balderrama et al. (2002). Electrode gel (Spectra 360, Parker Laboratories, Fairfield, NJ) was placed between the bee and the brass plates to ensure a continuous connection. A Velcro strap was secured across the thorax, restraining the bee while allowing for normal movement of the head and abdomen. Several restraints were used during the experiment and were cleaned after each bee to prevent a building up of alarm pheromone.

Testing

Once each bee recovered mobility, the Velcro strap restraint was situated in a specifically designed, plexiglass chamber (10″ h × 10′ 1 × 6” w) equipped to deliver a voltage under reproducible smoke conditions. Bees were placed inside the chamber, and connected to a Grass S88 stimulator (West Warwick, RI) with alligator clips clamped to the ends of the two brass plates (Fig. 1). The bee acclimated to the chamber for 30 s before the voltage was applied. Each bee received only a single voltage (either 1, 2, 4, or 8 V) for 2 s. These voltages were chosen to represent very mild to strong perturbations and were chosen based upon voltages applied during aversive learning experiments in honey bees (Balderrama et al. 2002, Vergoz et al. 2007).

Smoke was introduced into the chamber with a standard bee-smoker to replicate puffs of smoke delivered to a hive (Fig. 1). A hole, cut specifically for the bee-smoker was built into the side of the chamber. Once the bee had acclimated for 15 s, three puffs of a strongly smoking bee-smoker were applied to the chamber. The hole was closed, creating airtight smoke conditions. After 15 s of smoke exposure (30 s total in the chamber), the voltage was applied and behavior was scored. The release valve was removed after each bee and a vacuum exhaust fan pulled the smoke away from the chamber. All bees used for (-) No smoke conditions were tested first to prevent smoke contamination. (-) No smoke conditions were followed by (+) Burlap smoke, and then by (+) Hops smoke. After each condition, the chamber was cleaned with 70% ethyl alcohol followed by vacuum suction until evaporated. We did not observe changes in defensive behavior when a bee was introduced into the chamber for testing. Each active smoker was kept outside the testing area to prevent exposure to bees prior to testing.

Smoke type

Two types of material were used to generate smoke for the experiment. Burlap was used to create ‘(+’) Burlap’ conditions and is a common smoker material used in beekeeping. The other fuel used was spent hops pellets to create ‘(+’) Hops’ conditions (provided by J. I. Haas, Washington, DC, 9.5% moisture re-pelletized spent hops). This material is a new, alternative fuel source produced by female flowers of hops, and is a known sedative (Reilly 1906, Van Cleemput et al. 2009). Each smoker was stoked to provide 20 min of continuous smoke, the amount of time to test one group of 20 bees. With both fuel types, three puffs from a strongly smoking
bee-smoker were determined best to present to each bee, such that the chamber was filled with a thin haze of smoke, and the sting extension was fully visible (Figs. 1 and 2). Two brand-new smokers were used and designated for burlap or hops fuel exclusively to avoid contamination.

**Sting extension reflex**

We observed four distinct components of the sting extension reflex upon voltage application (Fig. 2). 1) The abdomen curl, which denotes the bee curling her abdomen toward her head, 2) tergite separation, where her sting chamber opens but the stinger does...
not protrude, 3) sting extension when the stinger protrudes, and 4) venom droplet release where a droplet appears on the stinger (Supp Video 1 [online only]).

Statistics
Wilcoxon/Kruskal–Wallis nonparametric tests were used to assess the effects of smoke on the sting extension response. A separate Wilcoxon was performed for each voltage level (1, 2, 4, and 8 V), followed by a Wilcoxon each pair post hoc analysis to assess smoke type differences. There was no significant difference between morning and afternoon experimental sessions for any of the four behaviors at any voltage level, and as a result morning and afternoon sessions were combined. For the venom droplet analyses, the incidence of release was measured in proportion to the number of sting extensions (ex. #droplets/#sting extensions). JMP 12.0.1 was used for all statistics, and all tests employed \( \alpha = 0.05 \). Error bars denote standard error mean.

Results
Abdomen Curl
The abdomen curl was observed immediately after voltage application in most bees (Table 1; Fig. 3). At the lowest level of perturbation (1 V) significant differences in abdomen curling were seen with smoke conditions. Bees in (+) Burlap smoke exhibited significantly more abdomen curls than bees in (−) No smoke conditions \( (P = 0.032, \text{Wilcoxon each pair}) \). Abdomen curls did not significantly differ between smoke conditions \( (P = 0.08, \text{Wilcoxon each pair}) \), though (+) Burlap elicited more abdomen curls (80%) than (+) Hops (62.5%).

At voltages 2, 4, and 8 V, there were no significant differences observed with smoke in the number of abdomen curls. At these voltages, abdomen curling was present in \( \geq 92\% \) of bees at each treatment and level of perturbation. At 2 V, control bees responded with abdomen curling to the stimulus 92.5% of the time, while each smoke condition generated a 95% response \( (X^2(2, N = 120) = 0.30, P = 0.86) \). Similar results were seen with 4 V \( (X^2(2, N = 120) = 0.51, P = 0.77) \) and 8 V \( (X^2(2, N = 120) = 1.01, P = 0.60) \) (Table 1; Fig. 3).

Tergite Separation
Instances of tergite separation increased with each step up in voltage and were not affected by smoke conditions (Table 1; Fig. 3). There was no significant effect of smoke on tergite separation at any voltage tested: 1 V: \( (X^2(2, N = 120) = 3.19, P = 0.202) \); 2 V: \( (X^2(2, N = 120) = 0.06, P = 0.97) \); 4 V: \( (X^2(2, N = 120) = 0.401, P = 0.82) \); or 8 V: \( (X^2(2, N = 120) = 0.71, P = 0.69) \).

Sting Extension
Similar to tergite separation, instances of sting extension also increased linearly with voltage under all conditions (Table 1; Fig. 3). There was no significant effect of smoke on the sting extension response at any voltage tested: 1 V: \( (X^2(2, N = 120) = 1.54, P = 0.46) \); 2 V: \( (X^2(2, N = 120) = 0.069, P = 0.97) \); 4 V: \( (X^2(2, N = 120) = 0.34, P = 0.84) \); or 8 V: \( (X^2(2, N = 120) = 0.64, P = 0.73) \).

Venom Droplet
During our first testing session using a 2 V stimulus, we noticed that some bees extended their stingers with a venom droplet while others did not. We monitored the release of the venom droplet throughout the remainder of the study. At 1 V, there were very few sting extension responses to examine [(−) No smoke: 6/40, (+) Burlap: 9/40, (+) Hops: 5/40]; and the incidence of droplets was fewer still [(−) No smoke: 2/40, (+) Burlap: 2/40, Hops: 0/40]. At this low level of perturbation, we did not see an effect of smoke on droplet release as a function of sting extensions \( (X^2(2, N = 20) = 1.84, P = 0.39) \) (Table 1; Fig. 4).

At 4 and 8 V, however, smoke did affect venom droplet release (Table 1; Fig. 4). At 4 V, both (+) Burlap smoke and (+) Hops smoke significantly reduced the likelihood of a venom droplet accompanying sting extension \( (X^2(2, N = 88) = 8.93, P = 0.012) \). This effect was comparable between smoke types \( (P = 0.76) \). At the highest perturbation level, 8 V, there was also an effect of smoke in reducing the probability of droplet release \( (X^2(2, N = 106) = 9.73, P = 0.0077) \). Only (+) Hops smoke showed this reduction, however. (+) Hops smoke resulted in significantly fewer droplets compared to (−) No smoke conditions \( (P = 0.0021, \text{Wilcoxon each pair}) \), and a reduction in venom droplet release compared to (+) Burlap smoke \( (P = 0.056, \text{Wilcoxon each pair}) \). At 8 V, (+) Burlap smoke did not differ from (−) No smoke conditions \( (P = 0.22, \text{Wilcoxon each pair}) \).

Discussion
We questioned whether the efficacy of smoke in reducing honey bee defensiveness was due to a reduction in the sting extension response. We hypothesized that smoke would reduce at least one component of the sting extension response in individual bees, and this effect would be most evident under lower levels (voltages) of perturbation. Four aspects of the sting extension response were evaluated: 1) Abdomen curl, 2) tergite separation, 3) sting extension, and 4) venom droplet release under two distinct smoke types: burlap and hops.

Overall, we found very few differences among abdomen curling, tergite separation, and sting extension between smoke and non-smoke conditions. The lowest perturbation level (1 V) showed the largest separation among treatments, though relatively minor. Bees exposed to burlap smoke showed slightly elevated defensive behavior compared with bees under nonsmoke, and hops conditions. This effect was significant with the abdomen curl measure. Burlap smoke

Table 1. Percentage of behavioral responses to increasing voltage steps under (−/+ ) smoke conditions

| Behavioral Response | Applied Voltage |
|---------------------|-----------------|
|                     | 1 V             | 2 V             | 4 V             | 8 V             |
| Abdomen curl        |                 |                 |                 |                 |
| (−) No smoke        | 57.50%          | 92.50%          | 97.50%          | 97.50%          |
| (+) Burlap          | 80.00%          | 95.00%          | 95.00%          | 100.00%         |
| (+) Hops            | 62.50%          | 95.00%          | 97.50%          | 97.50%          |
| Tergite separation  |                 |                 |                 |                 |
| (−) No smoke        | 17.50%          | 52.50%          | 82.50%          | 95.00%          |
| (+) Burlap          | 35.00%          | 50.00%          | 77.50%          | 92.50%          |
| (+) Hops            | 25.00%          | 50.00%          | 77.50%          | 90.00%          |
| Sting extension     |                 |                 |                 |                 |
| (−) No smoke        | 15.00%          | 40.00%          | 70.00%          | 90.00%          |
| (+) Burlap          | 22.50%          | 37.50%          | 75.00%          | 90.00%          |
| (+) Hops            | 12.50%          | 37.50%          | 75.00%          | 85.00%          |
| Venom droplet       |                 |                 |                 |                 |
| (−) No smoke        | 5.00%           | NA              | 37.50%          | 62.50%          |
| (+) Burlap          | 5.00%           | NA              | 15.00%          | 52.50%          |
| (+) Hops            | 0.00%           | NA              | 17.50%          | 30.00%          |

Each percentage denotes the proportion of 40 bees exhibiting a behavior. In total, 480 bees were tested.
produced significantly more abdomen curling than nonsmoke bees \( (P = 0.03) \), and more curling than hops-smoke bees \( (P = 0.08) \). It is somewhat counterintuitive to see elevated defensive behavior with burlap smoke, but it is possible that at this low perturbation, we captured the noxious effect beekeepers sense when puffing smoke into their hives. Bees scatter, and the audible volume of the hive briefly increases as smoke is applied. Smoke has been described as a disruptor to routine bee behavior (Graham 1992).

An unexpected variable in the sting extension response, the venom droplet release, may provide clues as to how smoke reduces the defensive response in agitated bees. Francois Huber in 1814 was the first to report that when he agitated bees in a semitorpid condition, they protruded their stings ‘upon which drops of venom appeared’ (Huber 1814). Maschwitz also wrote in 1964 that an agitated bee in an aggressive posture would extend her sting, and ‘sometimes a droplet appears on the tip’ (Maschwitz 1964). This measure was not determined prior to our study, but upon initial testing using a 2 V stimulus, the incidence of a venom droplet accompanying sting extension appeared to vary, and was carefully monitored throughout the study. We found that the venom droplet was more likely to be

![Fig. 3. Sting extension reflex behavior under smoke conditions. At each voltage level, 40 bees per group were randomly assigned to one of three conditions: (−) No smoke, (+) Burlap smoke, and (+) Hops smoke. Bees were assessed for (A) abdomen curl, (B) tergite separation, and (C) sting extension responses at 1, 2, 4, and 8 V. In total, 480 bees were used for this experiment. *Denotes significance between (−) No smoke and (+) Burlap smoke: \( P = 0.03 \), Wilcoxon each pair.](image-url)
released with greater perturbation; and the probability of its release was reduced with smoke. This finding may suggest that the release of the venom droplet coincides with greater defensiveness or agitation of individual bees. This release was reduced under smoke conditions at moderate and high levels of perturbation, even though the number of sting extensions matched nonsmoke controls. This raises the question of what constitutes the makeup of this droplet, and whether it may play a role in defensive communication among bees.

One of the hypotheses we pondered was whether there was a connection between alarm pheromone and the venom droplet release. Could the venom droplet, which suggests a stronger individual defensive response, also suggest a greater release of alarm pheromone? Is it possible that bees are less likely to release alarm pheromone under smoke conditions as a reason behind its efficacy? We researched the literature to piece together what is known about pheromone under smoke conditions as a reason behind its efficacy?

In 1962, Boch et al. identified isoamyl acetate (also known as isopentyl acetate) as an active component of the honey bee sting pheromone. Gunnison, in 1966, found that isoamyl acetate was present in the honey bee venom itself—separate from the motor apparatus and glands which would be removed with the sting in mammalian skin (Gunnison 1966). Gunnison collected venom by electrically stimulating honey bees in an effort to release ‘pure venom’ in a process (Gunnison 1966). Gunnison collected venom by electrically stimulating honey bees in an effort to release ‘pure venom’ in a process (Gunnison 1966). Morse and Benton, early authors of the ‘milking’ technique, soon published notes reporting that a common side effect of the venom extraction process was increased defensive behavior (Morse and Benton 1964). They wrote: ‘high [venom] yields themselves introduce complications, because of the large amounts of ‘alarm odor’ released at the same time as the venom’. They reported that previously docile hives that were ‘milked’ were difficult to work for 6–7 d after the process, and ‘angry bees were ready to sting anyone who came within a few hundred feet of the apiary’. In our study, we essentially employed the process of ‘milking’ by applying an electrical voltage to a bee. These observations of defensiveness with venom extraction support our finding of a positive correlation between increased venom droplet release with levels of perturbation. They also support the plausibility that venom droplet release may enhance alarm pheromone output.

More recently, we understand that the sting alarm substance is produced by the Koschevnikow glands and the proximal parts of the sting sheaths (Mauchamp and Grandperrin 1982, Cassier et al. 1994). The secreted alarm pheromone blend flows into the sting chamber, where it accumulates on the setaceous membrane (Mauchamp and Grandperrin 1982). The abundant setae on this membrane provide a large surface area enabling a quick discharge of pheromone whenever the sting is extruded (Lensky et al. 1995, Nouvian et al. 2016). We also know that honey bee venom includes pheromones, estimated to account for 4–8% of dried venom (Dotimas and Hider 1987). Isoamyl acetate was confirmed, as well as nine other volatile esters and alcohols which make up the alarm pheromone (Blum et al. 1978). Venom alone has shown mixed responses in eliciting stings from honey bees and this effect seems to hinge on whether the venom sac, venom gland, or pure venom is tested (Free and Simpson 1968, Cassier et al. 1994, Lensky et al. 1995). Pure venom has shown to elicit some stinging and attention from guard bees, but not as strongly as the setaceous membrane (Cassier et al. 1994, Lensky et al. 1995). From these findings, one might infer that alarm pheromone is dispersed from the setaceous membrane when the sting is extruded, and the release of the venom droplet may serve to amplify the sting alarm pheromone.

In this study, we sought to better understand how smoke application reduces the defensive behavior of honey bees. Smoke did not reduce abdomen curling, tergite separation, or sting extension, but did reduce the probability of venom droplet release. It is commonly thought that smoke masks alarm pheromone, but these findings suggest that smoke may instead minimize the release and spread. Further testing is necessary to understand whether venom droplet release equates with higher alarm pheromone levels; though observations of ‘angry bees’ after ‘milking’ and the existence of pheromones in the venom itself suggest this could be the case. We found...
that both burlap and hops smoke were effective in reducing venom droplet release, though hops smoke alone reduced its release at the highest perturbation. These results might be due to the properties of hops plants. Female hop cones contain glandular structures (lupulin glands) that secrete lupulin powder, rich in secondary metabolites classified as (resinous) bitter acids, volatile oils, and polyphenols (Van Cleemput et al. 2009). Hop extracts can reduce the excitability of the striated muscles and motor nerve endings, diminish irritability of the nervous system, and induce narcosis (Van Cleemput et al. 2009). Hops smoke, however, is notably denser than burlap, emitting a thick green haze. It is difficult to precisely control a smoke stimulus and differences in response to smoke type may be due to density or application differences rather than smoke composition. A limitation of this study, too, is that bees do not encounter electric shocks in nature, except under human activities involving venom extraction. A field study would need to be designed to assess these effects under more natural aversive conditions and with the social cues present in a hive setting. With these considerations, we propose that the presence of a venom droplet denotes greater agitation in individual bees and smoke functions to suppress honey bee defensiveness by reducing its release.

**Supplementary Data**

Supplementary data are available at *Journal of Insect Science* online.

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