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Playbacks of Asian honey bee stop signals demonstrate referential inhibitory communication

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Referential communication provides a sophisticated way in which animals can communicate information about their environment. Previously, research demonstrated that honey bee stop signals encode predator danger in their fundamental frequency and danger context in their duration. Here, we show that these signals also encode danger in their vibrational amplitude. Stop signals elicited by the more dangerous predator, the large hornet (*Vespa mandarinia*) had significantly 1.5-fold higher vibrational amplitudes than those elicited by the small hornet predator (*Vespa velutina*). We measured the freezing vibrational response thresholds, and show that natural signals exceed these response thresholds. Finally, with artificial playbacks of the vibratory stop signal, we demonstrate that these signals referentially encode the danger that foragers experience at food source. Stop signals elicited by the larger and significantly more dangerous predator (*V. mandarinia*) were significantly 1.4-fold more inhibitory than stop signals elicited by the smaller and less dangerous predator (*V. velutina*).

The coevolution of predator and prey has led to remarkable adaptations, including the ability to signal danger or even to warn of different danger levels. Referential communication encodes information about events or objects external to the signaller and can range from the sophisticated alarm system exhibited by vervet monkeys, which can warn of a specific predator types (Price et al., 2015), to far simpler antipredator calls (Blumstein, 1999). This kind of information transfer, sometimes termed information ecology, is important because it has cascading effects upon food webs (Brown, Laundré, & Gurung, 1999; Dall, Giraldeau, Olsson, McNamara, & Stephens, 2005; Laundré, Hernández, & Ripple, 2010; Orrock et al., 2008). For example, social bees are keystone species in multiple ecosystems because of the pollination services that they provide (Brown & Paxton, 2009), and their warning signals can influence predators and reduce pollination and plant fitness (Gonçalves-Souza, Omena, Souza, & Romero, 2008; Romero, Antiqueira, & Koricheva, 2011).

Honey bees can communicate food location with a waggle dance performed inside the nest that generates positive feedback when a returning forager recruits multiple foragers that, in turn, recruit other nestmates (Frisch, 1967). Such positive feedback is countered by another signal, the stop signal, which provides negative feedback by inhibiting the waggle dance (Nieh, 1993, 2010; Pastor & Seeley, 2005; Seeley et al., 2012) and reducing recruitment (Kirchner, 1993). Physically, the stop signal is a vibrational signal that is usually delivered by a bee butting its head against the body of a receiver and delivering a brief vibrational pulse, generated by the buzzing of its thoracic muscles (Michelsen, 2014). These signals have a fundamental frequency and duration of approximately 300–400 Hz and 150 ms, respectively, in *Apis mellifera* Linneaus 1758 (Lau & Nieh, 2010; Seeley et al., 2012) and 500–550 Hz and 178–258 ms, respectively, in *Apis cerana* Fabricius 1793 (Tan et al., 2016).

Stop signal receivers are most often waggle dancers (Nieh, 1993, 2010; Pastor & Seeley, 2005), but 18–30% of signals can be delivered to the comb (Thom, Gilley, & Tautz, 2003), often near waggle dancers. These stop signals are similar to a worker piping signal (Pratt, Kühnholz, Seeley, & Weidenmuller, 1996) and have also been called a 'brief piping signal' or a 'nectar forager pipe' (Pastor & Seeley, 2005; Thom et al., 2003). Previously, they were mistakenly thought to be a begging signal to elicit food exchange, but this rarely occurs: only in 0% (Pastor & Seeley, 2005) to 15% (Nieh, 1993)

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of cases. A key diagnostic of stop signals is that they cause the receiver to momentarily freeze during signal delivery (Kirchner, 1993; Michelsen, Kirchner, & Lindauer, 1986; Nieh, 1993; Pastor & Seeley, 2005; Seeley et al., 2012; Towne, 1985).

The stop signal appears to primarily inhibit waggle dancing (Nieh, 2010; Seeley et al., 2012; Tan et al., 2016). Conditions at food sources can rapidly deteriorate, and stop signals help update the colony. Foragers that experience adverse food conditions (Lau & Nieh, 2010; Nieh, 1993; Thom, 2003) or that are attacked by predators (Jack-McCollough & Nieh, 2015; Tan et al., 2016) or conspecifics (Nieh, 2010) direct stop signals at foragers recruiting for these now dangerous food sources (Nieh, 2010). Stop signals could therefore have an ecosystem impact upon pollination. During house hunting, bees advertising different sites also deliver stop signals to speed up decision making via cross-inhibition (Seeley et al., 2012). In the context of foraging, stop signals elicited by danger function as alarm signals. However, the broadest interpretation, one that includes their use in house hunting, is that the stop signal is an inhibitory signal (Seeley et al., 2012).

The Asian honey bee, *A. cerana*, is an excellent model for studying such inhibitory signalling. *Apis cerana* is an important pollinator of native Asian plants (Corlett, 2001; Huang, 2005) and plays this role over a wide range of ecosystems: it occurs throughout southern and eastern Asia, with a geographical range extending from India to China and Japan (Peng, Nasr, & Locke, 1989). *Apis cerana*, remarkably, encodes predator threat levels, countering the referential excitatory signal of the waggle dance with a referential inhibitory signal, the stop signal (Tan et al., 2016). Attacks upon foragers at the food source by the large hornet *Vespa mandarinia* Smith 1852 elicit stop signals with a significantly higher frequency (550 Hz) than attacks by the smaller hornet *Vespa velutina* Lepeletier 1836.

The large hornet is more dangerous than the small hornet because it inflicts 13-fold higher mortality on colonies (Tan et al., 2016). Foragers show appropriate responses, and their dancing is more strongly inhibited by stop signals elicited by the large hornet (SS_{LH}) than those elicited by the small hornet (SS_{SH}). *Apis cerana* stop signals therefore show a level of sophistication that is greater than has been shown in any subsocial or social insect. Treehoppers (Hamel & Cocroft, 2012), ants (Pielström & Roces, 2012) and termites (Hager & Kirchner, 2013) can use vibrations to signal alarm. Cyprian honey bees, *A. mellifera cypria*, produce alarm signals, hissing sounds when attacked by the Oriental hornet, *Vespa orientalis* (Papachristoforou et al., 2008). Alarmed *Apis florea* workers can deliver brief piping signals to the comb (Sarma, Fuchs, Werber, & Tautz, 2002). *Apis mellifera* guard bees attacked by the hornet *Vespa simillima* produce piping signals (Ohtani & Kamada, 1980). However, none of these signals are known to be referential.

In *A. cerana*, predators may release other acoustic signals: piping-hissing responses to predators have been reported (Fuchs & Radloff, 2011). Hissing sounds can also be elicited by vibrating or tapping the *A. cerana* comb at frequencies <600 Hz (Fuchs & Tautz, 2011). Other Asian honey bees also possess such vibrational signals. Towne (1985) reported stop signals in *Apis dorsata* (375 Hz) and *A. florea* (475 Hz). Sarma, Sen, Fuchs, Werber, and Tautz (2002) wrote that alarmed *A. florea* workers would deliver brief piping signals (mean ± 1 SD: fundamental frequency: 384 ± 31 Hz; duration: 0.82 ± 0.35 s) to the comb. These signals elicited a wave of hissing behaviour by other bees that spread through the comb and restricted colony activity, such as waggle dancing and departures from the colony.

We previously showed that stop signals produced by *A. cerana* foragers that had been attacked by hornets could elicit appropriate responses in signal recipients that had not encountered these predators (Tan et al., 2016). However, although signal producers and recipients fed at different feeders, attacked bees may have carried back the predator's odour into the nest, and this could have influenced receiver responses. To eliminate the effects of hornet odour, we therefore used artificially generated playbacks to test the hypothesis that these signals are referential.

We also wished to understand why SS_{LH} are more inhibitory than SS_{SH}. SS_{LH} have a higher frequency (550 Hz) and inhibit waggle dances more strongly than SS_{SH} (500 Hz) (Tan et al., 2016). In *A. mellifera*, Michelsen (1986) investigated worker responses to a wide range of stop signals and demonstrated that workers have decreased response thresholds for higher-frequency signals. We hypothesized that higher-frequency stop signals are inherently more inhibitory (1) because receivers are more sensitive to higher-frequency signals. However, a stronger inhibitory response could also arise (2) if SS_{LH} have a significantly higher vibrational amplitude than SS_{SH}, or (3) if both frequency and amplitude play a role. We therefore determined the freezing response thresholds of *A. cerana* workers to comb vibrations and measured the vibrational amplitudes of stop signals elicited by *V. velutina* and *V. mandarinia*.

METHODS

General Methods

Three *A. cerana cerana* colonies were housed in observation hives in a room at Yunnan Agricultural University, Kunming, China during July–September 2016, July 2017 and October 2018 and observed between 09:00 and 15:00 hours. Each observation hive (55.4 × 17 × 64 cm) contained two combs (43.5 × 23 cm) and was connected to the outside with a tube (2.2 cm inner diameter, 25 cm long) piercing the wall. To view all dance activity, we used a wood and beeswax divider to direct all bees to the lower comb on one side of the nest (method of Nieh, 1993) where the dance floor was located (Tan et al., 2012). We removed the glass covering the dance floor side of the nest to record bee sounds. Bees could exit via a door in the observation hive room, but foragers rapidly acclimated to the opened hive and soon entered and left by its tubular entrance. We used Liebefelder photographic estimation (Imdorf, Buehlmann, Gerig, Kilchenmann, & Wille, 1987) and determined that each colony contained approximately 5000 bees.

We trained nestmates, five at a time, to a 50% (w/w) sucrose solution feeder located 100 m from the focal colony by gently capturing departing foragers at the hive entrance in vials (one bee per vial) and releasing them slowly at the training feeder. Bees from each colony were trained to a different location to avoid competition for the same food source. All bees were individually marked with different colour combinations of acrylic paint on their thorax. The feeder consisted of a 70 ml vial (8 cm high) inverted over a circular plastic disk with 18 feeding grooves through which the sucrose could flow. After being filled with sucrose solution, the vial was inverted over the grooved plate and placed on a blue plastic square to facilitate visual recognition. Each feeder could accommodate 30 foragers without crowding. However, we only allowed five bees to feed at a time, capturing excess foragers with an aspirator. The feeder and sucrose solutions had no scents added. No predators were ever present at any feeder or at the nest during our experiments. We conducted three different types of playback experiments, each with a different set of foragers. In each case, we randomly selected a focal bee and waited for her to return inside the nest, unload her collected food and begin to waggle dance. In all playback experiments, the behaviour of each bee was recorded during only one trip inside the nest. After this trip, the bee was removed upon returning to the feeder with an aspirator. In some experiments (see below) bee behaviours were video-recorded (Sony HDR-PJ790 camera) for scoring by blinded observers.

Experiment 1: Freezing Response Threshold

We measured the freezing response threshold to sine wave playbacks over a range of frequencies and amplitudes, recording the minimal amplitude required at each frequency to elicit freezing by comb bees. We embedded an accelerometer (Brüel & Kjaer miniature accelerometer type 4393 connected to a B&K charge amplifier type 2635) in the centre of the dance floor on the lower comb (see Fig. 1) where most stop signals elicited by predator attacks at food sources are produced (Tan et al., 2016). The comb was attached only at the top and was free on the three other sides to facilitate vibrational propagation (Tautz & Rohrseitz, 1998). We used function generator software (AudioTest v.2.2, Katsura Shareware, http://www.katsurashareware.com/) to play back vibrations through our playback probe (see above), attached with beeswax to the lowest point on the comb centre (Fig. 1). We considered freezing to occur when the majority (>50%) of bees on the lower comb remained motionless. To control for potential observer bias, we additionally ran a series of blind trials (sample size in Fig. 1), using a limited set of frequencies (200, 300, 500, 550 and 1000 Hz) identified from the original nonblinded experiment. The experimenter played back these frequencies at different, randomized amplitudes and videorecorded the results, visually indicating on the video when a change was made. Separately, an observer blind to the actual treatment watched and scored the silent videos.

Experiment 2: Recording Vibrational Amplitudes and Calibration

We trained bees that were painted with different colours on their thoraces for identification to an unscented inverted dish feeder placed 100 m from the focal colony. To elicit stop signals, we attached a hornet (*V. velutina* or *V. mandarinia*) to a clean 1 m long wooden stick by wrapping wire around its petiole and the end of the stick. The feeder monitor then attacked focal foragers by gently touching a feeding bee with a hornet for < 1 s while ensuring that the bee would escape and not be harmed by the hornet, which could impair its ability to fly back to the colony. Bees thus contacted immediately fled and returned to the nest (Tan et al., 2016).

The amplitude of natural stop signals produced by bees was recorded by screwing a B&K miniature accelerometer type 4393 (reference sensitivity of 03169 pC per m per s) to a 20 cm wooden rod (total wand mass of 6 g) connected to a charge amplifier type 2635. The accelerometer was calibrated with a B&K calibration exciter type 4294. This vibration-recording wand was then gently placed next to foragers that had been attacked at the feeder and were producing stop signals, as detected with a shielded pressure microphone (Movo LV1 Lavalier Microphone) mounted on a thin metal rod. The microphone output was connected to a RadioShack Mini Amplifier (model 277-1008) and monitored with headphones to provide the experimenter with immediate feedback on whether a bee was producing stop signals. The accelerometer output was recorded with an ASUS K53S computer and a digital oscilloscope (Rigol Model DS1054).

Because we followed stop-signalling bees as they moved around on the comb, they would sometimes directly deliver a stop signal to the accelerometer, allowing us to measure signal amplitude. Occasionally, bees would also give stop signals to the comb near a dancing bee. However, we only measured stop signals in which the bee made direct contact between its head and the top of the accelerometer, corresponding to the device's axis of maximum sensitivity (perpendicular to the mounting plane). We focused on head contact because bees typically deliver the stop signal by butting their head against a receiver or the substrate (Nieh, 1993).

We only recorded the signals generated by bees. We did not record any vibrations or signals produced by hornets. We recorded stop signals elicited by the small hornet (SS_{SH}, *V. velutina*) and, using different bees, by the large hornet (SS_{LH}, *V. mandarinia*). Signal amplitudes were directly measured from oscilloscope recordings, and the average amplitude was used for our playback experiments. Tan et al. (2016) previously reported detailed signal fundamental frequencies and pulse durations for SS_{SH} (500 ± 10 Hz, 170 ± 9 ms) and SS_{LH} (550 ± 4 Hz (180 ± 4 ms).

We also ran a set of blind trials (120 bees from three colonies) in which the experimenter attacked bees at the feeder with a randomly selected *V. velutina* or *V. mandarinia* while an observer who was unaware of the type of attacker measured the amplitudes of stop signals produced by the attacked bees.

Our accelerometer measurement method is less sensitive than using a laser Doppler vibrometer, but it has two main advantages. First, it is difficult, although possible (Hrncir et al., 2006), to track a rapidly moving bee with the laser. In our experience, signal artefacts from the experimenter moving the laser and the bee moving on the comb often resulted in unusably noisy signals. Second, stop signals can occur over a wide area and the wand can be easily and rapidly moved to the location of the signaller, whereas redirecting a stably mounted laser to track a rapidly moving target is more challenging.

Playback Device

The playback device consisted of a simple wand design (Fig. 1), following the model of Nieh (1993). To a lightweight wooden rod (5 cm diameter and 40 cm long), we attached an 8 Ohm, 2 W speaker (36 mm diameter) that had a 10 µl pipette tip (Fisherbrand, Cat. No. 21-197-2E) attached and trimmed to a height of 25 mm with a tip diameter of 2 mm. To this tip, we attached a 3.6 mm diameter ball of beeswax (obtained from the nest under study) to stimulate the head of a bee delivering the signal. Thus, the motion of the speaker cone was converted to vibration along the vertical axis of the attached tip. The signal was delivered to the miniature speaker via wires controlled by a miniature momentary-on push button switch at the base of the rod. A cable connected to a 3.5 mm audio jack at the wand base connected the device to a custom-built low-noise 10× amplifier and to an ASUS K53S computer that played back sounds via its audio output port. We chose this design because it was lightweight and it allowed the researcher to rapidly move the wand to target each bee precisely without applying excessive pressure. Preliminary trials with a Bruel & Kjaer mini shaker type 4810 were not successful because even this small shaker (1.1 kg) was so heavy that an experimenter trying to agilely follow and contact a rapidly moving waggle dancer or the immediately adjacent comb usually pushed the dancer against the comb, eliciting a stinging response or permanently deforming the comb.

A representative natural signal was chosen for SS_{SH} and for SS_{LH}. These signals were duplicated and adjusted in amplitude using Raven v.1.4 software (Cornell Lab of Ornithology, Ithaca, NY, U.S.A.) to generate looped playback sequences. The computer played back one signal per second, and, in conjunction with the momentary switch, the researcher could easily control signal delivery.

To calibrate playbacks, we placed the signal-delivery probe in contact with the B&K miniature accelerometer type 4393 for playbacks delivered to bees. For playbacks delivered to the comb, we embedded this accelerometer in the comb, 4.4 mm (1 cell diameter) away from the probe that also contacted the comb. In both cases, we oriented the accelerometer so that its sensitivity axis was aligned with the vibrations delivered by the probe. The probe was calibrated to deliver the following naturally recorded stop signals: SS_{SH} (fundamental frequency = 500 Hz, amplitude = 5.9 m/s^2) and SS_{LH} (fundamental frequency = 550 Hz, amplitude = 9.7 m/s^2). These

amplitudes were based upon our measurements of natural SS_{LH} and SS_{SH} (see Results). Subsequently, we used a laser Doppler vibrometer (Polytec OFV3000 controller unit with an OFV502 laser head) and confirmed this calibration of the playback wand.

This probe produced no detectable particle velocity sound at the probe tip located 25 mm from the speaker surface. To measure particle velocity sound produced by our device, we used a Microflown particle velocity scanning probe (ST0905-45) and calibrated signal conditioner (E0905-45). This Micro-Electro-Mechanical System (MEMS) sensor directly measures acoustic particle velocity (Hrncir, Schorkopf, Schmidt, Zucchi, & Barth, 2008; Tsujiuchi, Sivan-Loukianova, Eberl, Kitagawa, & Kadowaki, 2007). When playing back stop signals, our device produced particle velocity at amplitudes of 0 ± 0 mm/s (25 mm above speaker, at the probe tip), 9.6 ± 1.2 mm/s (1 mm above speaker) and 5.6 ± 2.5 mm/s (5 mm above speaker). In honey bees, the Johnston's Organ detects particle velocity sound and, for frequencies around 265 Hz, requires particle velocity displacements greater than 5–6.7 mm/s to elicit measurable, sound-evoked electrophysiological potentials (*A. mellifera*: Tsujiuchi et al., 2007). For comparison, Michener (1987) measured maximum particle velocity displacements of 500 mm/s at 1 mm from the vibrating wing tips of waggle dancers. Thus, honey bee workers would need to be about 5 mm from the vibrating speaker surface to detect particle velocity sound. However, they were always at least $\geq$25 mm away, a distance at which our device produced no measurable particle velocity sound. For bees, the only detectable sounds produced by the playback wand should therefore be substrate vibrations.

In all playback experiments, signals were only played back when no other trained bees were on the dance floor. Each bee only received one type of signal and was not reused for subsequent playbacks.

Experiment 3: Bee versus Comb Playbacks

We were concerned about the potentially disruptive effect of the probe contacting a bee. We therefore first tested the efficacy of playbacks that contacted the waggle dancer (direct contact) as compared to the comb immediately adjacent to the waggle dancer. Direct contact playbacks consisted of the experimenter lightly touching the tip of the probe to the abdomen (either left or right side, randomly selected) and administering a single SS_{SH} or SS_{LH} to a waggle dancer. Comb playbacks consisted of the experimenter placing the probe tip in contact with the closest comb cell wall to the waggle dancer, approximately 4.4 mm (Yang, Tan, Radloff, Phiancharoen, & Hepburn, 2010) away from the edge of the dancer's abdomen and delivering either a SS_{SH} or SS_{LH}. During the signal playback, the bee was scored as freezing or not freezing (moving). Because bees clearly froze in response to comb playbacks, not direct contact (perhaps because of the disruptive nature of direct contact with the probe), we only used comb playbacks (1 cell width away, 4.4 mm, from the abdomen of the dancer) in all subsequent experiments. For each bee, we first recorded her dance behaviour in the nest after she had fed undisturbed at the feeder ('before phase') and then after she had been attacked by a hornet at the feeder ('after phase').

Experiment 4: Fixed-signal Playbacks

The two-signal playbacks consisted of the experimenter delivering two stop signals (1 s apart) to a bee immediately after it completed one waggle dance circuit. We used two stop signals because this is the average number of stop signals received per dance performance by foragers for natural food sources at our research site (Tan et al., 2016). We then counted (and videorecorded) the total number of waggle dance circuits that she produced during the remainder of her visit inside the nest (one dancing bout). We recorded the dance behaviour of each bee in two phases, before and after attack, as in experiment 3.

To control for potential bias, we conducted an additional 120 blind playbacks (40 to each of the same three colonies) in which the experimenter conducting the playback did not know and could not hear the type of stop signal being played back. These trials were videorecorded. Subsequently, a different observer who was also blind to the treatment watched the silent videos and counted the number of dance circuits performed by each dancer.

Experiment 5: Multi-signal Playbacks

Prior studies of individual bees have examined the effects of naturally distributed stop signals (Nieh, 2010; Seeley et al., 2012) or a single stop signal (Nieh, 1993). However, the effects of repeatedly receiving multiple signals has not been tested. We therefore conducted multi-signal playbacks following the same procedure as the fixed-signal playbacks, except that we continued to deliver stop signals (1 per dance circuit) until the dancer stopped dancing. For example, a dancer that produced 10 dance circuits would receive 10 signals. We counted the total number of waggle circuits during a forager's visit to the nest, as in experiment 4.

Statistics

To analyse the differences between the log-transformed amplitudes of SS_{SH} versus SS_{LH}, we used ANOVA and an REML algorithm with colony as a random effect. For the freezing substrate data, we used nominal logistic fit models with colony as a fixed effect and report likelihood ratio (L-R) chi-square test results. To analyse the effect of stop signals on waggle dancing, we used repeated measures analysis of variance (ANOVA, REML algorithm) with colony as a random effect. We log transformed unloading and dance duration times. To determine the effect of two stop signals delivered to a waggle dancer, we log transformed the number of waggle circuits and unloading duration and used a repeated measures ANOVA. To test the number of stop signals required to stop waggle dancing, we log transformed the number of stop signals. Colony was a random effect in all models unless otherwise specified. All other effects were fixed. In experiments with blinded treatments, experiment type (blinded or nonblind) was also a fixed effect. We used stepwise model simplification. Post hoc comparisons were made with Tukey honestly significant difference (HSD) tests. We used JMP Pro v14.0.0 statistical software (SAS Institute Inc., Cary, NC, U.S.A.) for all analyses. We report mean $\pm$ 1 SE. All samples sizes are shown in the figure legends.

RESULTS

Freezing Response Thresholds Decrease with Higher Frequencies

As expected, bees were more sensitive to higher frequencies, and freezing response thresholds declined as frequency increased (Fig. 1). The frequencies of natural stop signals (500 Hz for SS_{SH} and 550 Hz for SS_{LH}) occupy the most sensitive part of the vibrational acceleration threshold. Specifically, the freezing thresholds were 0.63 ± 0.25 m/s^2 (at 500 Hz, SS_{SH}) and 0.58 ± 0.28 m/s^2 (at 550 Hz, SS_{LH}). Blind and nonblind observations were not significantly different (experiment type: $F_{1,4} = 0.01$, $P = 0.93$), but there was, as expected, a significant effect of frequency ($F_{4,115} = 11.58$, $P < 0.0001$) because the response threshold was lower for higher frequencies (Fig. 1). The interaction of experiment type $*$ frequency was not significant ($F_{4,111} = 0.004$, $P > 0.99$), and colony accounted for 49% of model variance.

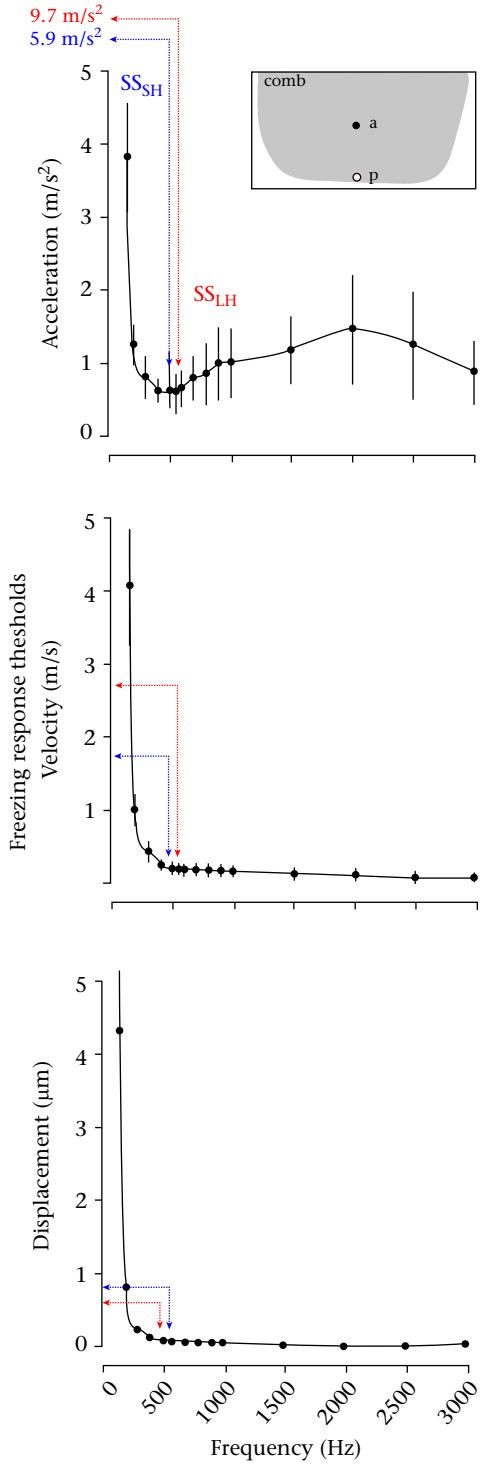

Figure 1. Freezing response thresholds of bees in response to whole comb vibrations. Sinusoidal frequencies were delivered via a playback probe placed at the centre bottom of a freely hanging comb where the dance floor was located, and an accelerometer measured vibration amplitudes (see inset: p = probe; a = accelerometer). The threshold was reached when a majority of bees on the comb (>50%) exhibited a freezing response. Arrowheads and dashed lines indicate mean amplitudes and corresponding mean frequencies of SS_{SH} (stop signals elicited by small hornets, blue) and SS_{LH} (stop signals elicited by large hornets, red), respectively (see also Fig. 2). Data are from eight trials per colony with three colonies (divided equally into blind and nonblind data, but pooled here because there was no significant effect of experiment type). Means and standard errors are shown.

SS_{LH} Have Significantly Higher Amplitudes than SS_{SH}

Attacks by the large hornet caused foragers to produce stop signals that were significantly 1.5-fold higher in vibrational amplitude (peak-to-peak displacement) than the small hornet ($F_{1,311} = 58.78$, $P < 0.0001$; Fig. 2). There was no effect of experiment type (blind versus nonblind: $F_{1,311} = 0.001$, $P = 0.97$) and no significant interaction of experiment type∗hornet species ($F_{1,310} = 0.002$, $P = 0.96$). Colony accounted for 4% of model variance. Mean stop signal amplitudes (acceleration, velocity, displacement) for SS_{SH} and SS_{LH} all exceeded the freezing response thresholds: 5.9 m/s^2 (9.4-fold higher than threshold, corresponding to the fundamental frequency of SS_{SH}) and 9.7 m/s^2 (16.7-fold higher than the threshold for SS_{LH}, Fig. 1).

Comb Playbacks, But Not Direct Contact Playbacks, Elicited Freezing

Comb playbacks were more effective than direct bee-contact playbacks. We played back recorded SS_{SH} and SS_{LH} at their mean natural amplitudes (shown in Fig. 2). In response to direct contact with the playback probe, bees sometimes appeared disturbed and moved away from the probe tip. Such direct playbacks did not cause freezing (L-R: playback type: $\chi^2_3 = 0.76$, $P = 0.86$; colony: $\chi^2_2 = 5.06$, $P = 0.08$; Fig. 3a). However, playbacks directed at the comb did cause freezing (playback type: $\chi^2_3 = 634.22$, $P < 0.0001$; colony: $\chi^2_2 = 11.31$, $P = 0.004$; Fig. 3b) and bees did not move away from the probe tip. There was a significant colony effect because the freezing response was slightly greater in some colonies. When analysed individually, all colonies showed significantly greater freezing in response to comb playbacks (playback type: $\chi^2_3 \geq 187.54$, $P < 0.0001$). There was no significant difference between the efficacy of SS_{LH} or SS_{SH} comb playbacks at eliciting freezing (playback type: $\chi^2_1 = 1.92$, $P = 0.17$; colony: $\chi^2_2 = 3.04$, $P = 0.22$), as expected given that both playbacks were above the freezing response thresholds.

SS_{LH} Were Significantly More Inhibitory than SS_{SH}

We next played back stop signals to waggle dancers (two signals per dance performance). Stop signals reduced waggle dancing

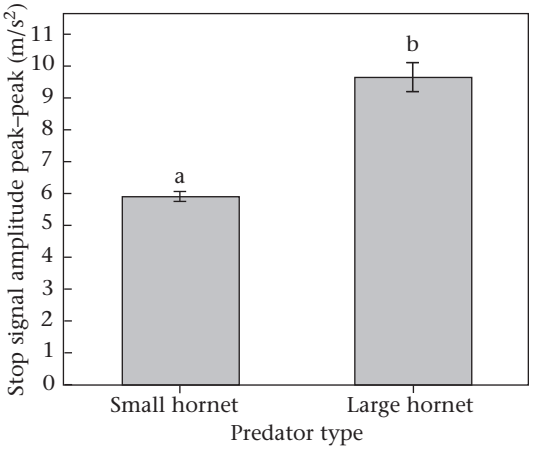

Figure 2. Honey bee stop signal amplitude (peak–peak acceleration) in response to large hornet (LH) and small hornet (SH) attacks. Means and standard errors are shown. On average, these stop signals had velocities of 1.8 and 2.8 m/s and displacements of 0.6 and 0.8 µm for SS_{SH} (500 Hz) and SS_{LH} (550 Hz), respectively. Different letters indicate significant differences. Each signal is from a different bee. For the nonblind data, sample sizes for SS_{LH} are $N = 30$, 30 and 46 signals from colonies 1, 2 and 3, respectively, and for SS_{SH} are $N = 30$, 29 and 31 signals from colonies 1, 2 and 3, respectively. For the blind data, we recorded 20 stop signals per colony per hornet species. These data are pooled because there was no effect of experiment type.

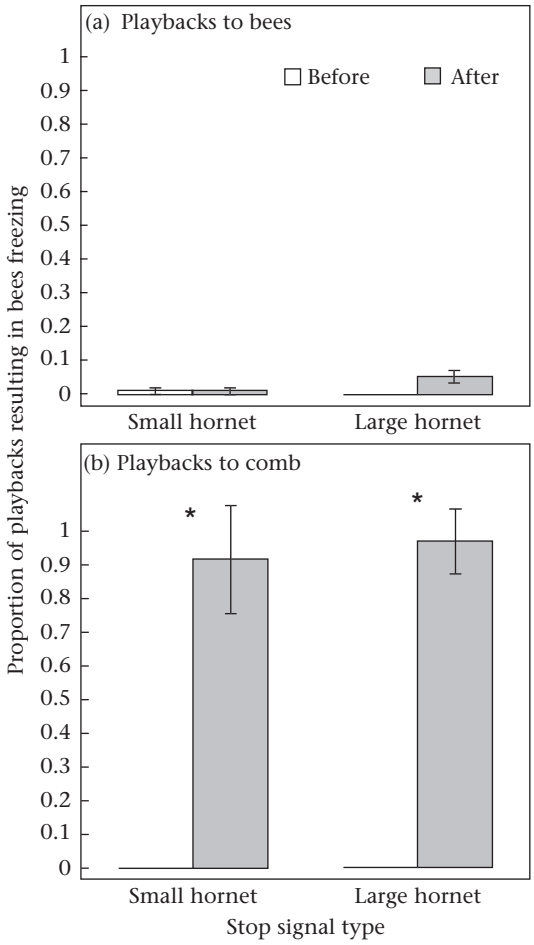

Figure 3. Effect of stop signal playbacks targeting (a) individual bees or (b) the comb on bee freezing responses. Sample sizes for the bee substrate playbacks are $N_{\text{small hornet SS}} = 133$ (35, 49 and 49 bees, respectively, from colonies c1, c2 and c3) and $N_{\text{large hornet SS}} = 140$ (42, 49 and 49 bees). Sample sizes for comb substrate playbacks are $N_{\text{small hornet SS}} = 132$ (34, 49 and 49 bees) and $N_{\text{large hornet SS}} = 140$ (42, 49 and 49 bees). Means and standard errors are shown. Asterisks indicate significant differences between the before and after phases ($P < 0.05$).

(phase effect: $F_{1,118} = 336.90$, $P < 0.0001$), and stop signals elicited by the large hornet (SS_{LH}) were significantly more inhibitory than those elicited by the small hornet (SS_{SH}): $F_{1,115} = 7.83$, $P = 0.006$ (Fig. 4). There was no significant effect of experiment type (blind versus nonblind: $F_{1,115} = 22.03$, $P < 0.0001$). There was a significant interaction stop signal type∗phase ($F_{1,118} = 22.03$, $P < 0.0001$) because SS_{LH} were more inhibitory than SS_{SH} (Tukey's HSD test: $P < 0.05$). All other interactions were nonsignificant ($F_{1,114\text{-}116} \leq 1.13$, $P \geq 0.29$). Colony accounted for <1% of model variance.

Unloading wait time can influence bee behaviours (Kirchner, 1993; Seeley, 1992). However, our waggle dancers had similar unloading times (21.7 ± 0.6 s) during all phases and treatments: there was no effect of stop signal type ($F_{1,56} = 0.07$, $P = 0.79$), phase ($F_{1,58} = 2.28$, $P = 0.14$), or the interaction stop signal type∗phase ($F_{1,58} = 0.15$, $P = 0.70$). Colony accounted for <1% of model variance.

Fewer SS_{LH} Were Required to Stop Waggle Dancing than SS_{SH}

In this experiment, we played back multiple stop signals until the foragers ceased waggle dancing. Again, SS_{SH} were less inhibitory than SS_{LH} because significantly more SS_{SH} were required to stop waggle dancing than SS_{LH} ($F_{1,116} = 46.31$, $P < 0.0001$; Fig. 5). Our data also allowed us to estimate how many stop signals of each

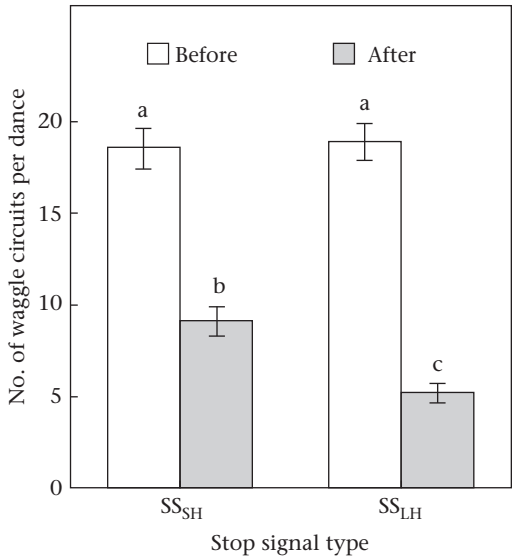

Figure 4. Effect of stop signal playbacks on waggle dancing (experiment 4: two stop signal playbacks per waggle dancer). Different letters indicate significant differences (Tukey's HSD tests). Means and standard errors are shown. We played back stop signals to 240 different bees (20 per category), divided equally among the two stop signal types (SS_{LH} and SS_{SH}), experiment types (blind and nonblind, pooled here because there was no significant effect of experiment type) and the three colonies.

type were required to stop waggle dancing. For SS_{SH}, the slope was 2.1 SS_{SH}/waggle dance circuit (linear regression: $R^2 = 0.82$, $t = 16.14$, $P < 0.0001$). For SS_{LH}, the slope was 1.1 SS_{LH}/waggle dance circuit ($R^2 = 0.92$, $t = 25.91$, $P < 0.0001$).

All waggle dancers also had similar unloading times (19.9 ± 0.4 s) during all phases and treatments. There was no effect of stop signal type ($F_{1,117} = 3.68$, $P = 0.06$), phase ($F_{1,119} = 3.09$, $P = 0.08$), or the interaction stop signal type∗phase ($F_{1,119} = 0.65$, $P = 0.42$) on unloading times. Colony accounted for 6% of model variance.

DISCUSSION

The coevolution of *A. cerana* and its hornet predators has led to a sophisticated signalling adaptation. By playing back calibrated,

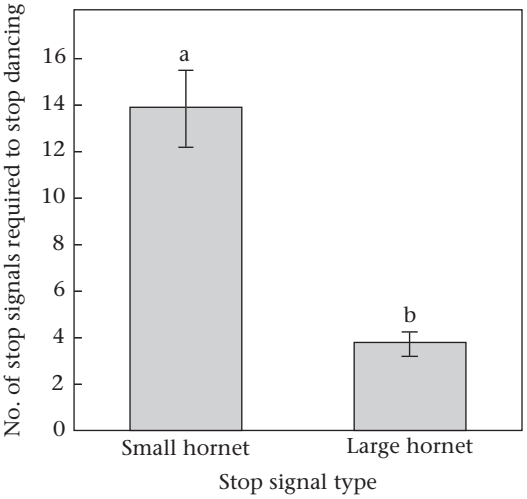

Figure 5. Number of stop signals required to stop waggle dancing (experiment 5: multiple stop signal played back until the waggle dancer stopped dancing). Different letters indicate significant differences (Tukey's HSD tests). Means and standard errors are shown (bee substrate playbacks: $N_{\text{small hornet SS}} = 60$ and $N_{\text{large hornet SS}} = 60$; equally divided among three colonies).

artificially generated stop signals that were free of any predator cues or signals, we confirmed that *A. cerana* stop signals are inhibitory and referential. Stop signals (SS_{LH}) elicited by the large hornet (*V. mandarinia*) reduced the average number of waggle dance circuits by 72%, and stop signals (SS_{SH}) elicited by the small hornet (*V. velutina*) reduced waggle dancing by 51%. In the fixed-signal playbacks, SS_{LH} were thus 1.4-fold more inhibitory than SS_{SH}. The multi-signal playbacks yielded a similar result, with 3.7-fold more SS_{SH} required to stop dancing than SS_{LH}. Our measurements of signal amplitude suggest an explanation for the greater inhibitory power of SS_{LH}. Stop signals elicited by the more dangerous predator, the large hornet, had significantly 1.5-fold higher vibrational amplitudes than those elicited by the small hornet predator, closely matching the observed 1.4-fold increase in inhibition.

Variation in how the experimenter held the measuring accelerometer potentially influenced the accuracy and repeatability of our amplitude measurements: the coefficients of variation were 0.60 and 0.30 for SS_{LH} and SS_{SH}, respectively. However, the difference in signal amplitudes between SS_{LH} and S_{SH} was large and highly significant, and there was no significant difference between the blind and nonblind data. Our measured *A. cerana* stop signal amplitudes are also comparable to those reported for *A. mellifera* stop signals, which vibrate the comb with a peak-to-peak displacement of 1.5 μm at 320 Hz (Michelsen et al., 1986). *Apis cerana* is a smaller bee and their signals are lower in amplitude, as might be expected for lighter bees: SS_{SH} (peak–peak = 0.6 μm at 500 Hz) and SS_{LH} (peak–peak = 0.8 μm at 550 Hz). We weighed exiting foragers from the nest entrances of three colonies of each species in our apiary (10 bees per colony) and found that *A. mellifera* foragers were 1.4-fold heavier than *A. cerana* foragers: *A. mellifera* (87.7 ± 1.4 mg) and *A. cerana* (62.1 ± 1.4 mg).

Apis cerana is evidently more sensitive to the disturbance of contact with a vibrating probe tip than *A. mellifera* (Nieh, 1993). Vibration paired with contact may be the disrupting factor since bees did not exhibit disturbance when touched with the non-vibrating recording probe. However, playbacks to the comb next to a dancer evidently did not disturb bees and were effective at eliciting freezing (Fig. 3) and decreasing waggle dancing (Figs 4 and 5). Such playbacks have a natural context because we also observed natural stop signal delivery to the comb immediately next to a dancer. In multiple cases, stop signallers seemed to be targeting a waggle dancer because they delivered multiple consecutive signals at the same dancer but occasionally appeared to miss their rapidly moving target and therefore contacted the comb or a nearby bee.

However, variation in how the experimenter held the playback wand could have influenced our results. We wished to replicate a stop signaller targeting a rapidly moving waggle dancer that could dance over a wide area of comb, and a moving playback device was therefore necessary. Because of this potential variation, we used a large sample size (targeting 240 bees from three colonies) and conducted blind trials in which the experimenter did not know what kind of playback was being delivered. There was no significant difference between blind and nonblind data: SS_{LH} remained significantly more inhibitory than SS_{SH}.

But why are SS_{LH} more inhibitory than SS_{SH}? The freezing response threshold data revealed lower freezing thresholds in response to higher frequencies. However, the response threshold difference between 550 Hz (SS_{LH}) and 500 Hz (SS_{SH}) was quite small (a 7% decrease, Fig. 1) and, in any case, natural stop signals significantly exceeded receiver response thresholds. These data do not strongly support the frequency hypothesis (H1). Instead, SS_{LH} are likely more inhibitory simply because they have a higher amplitude than SS_{SH} (H2). Interestingly, the 1.5-fold higher vibrational amplitudes of SS_{LH} versus SS_{SH} closely matches the observed 1.4-fold increase in inhibition. Both frequency and amplitude play a role (H3), but testing this requires additional testing.

Stop signals appear to be a kind of honey bee worker piping signal because they share a similar generation mechanism, vibrations of the wing muscles, and are often delivered to the substrate (Armbruster, 1922; Ohtani & Kamada, 1980; Orosi-Pal, 1932). There are multiple reports of such piping signals. Ohtani and Kamada (1980) noted that egg-laying workers in queenless nests produced lower-frequency piping (350 Hz) and guard bees attacked by hornets gave higher-frequency signals (500–700 Hz). Pratt et al. (1996) reported that *A. mellifera* foragers (including pollen or water foragers) would sometimes return to the nest and produce a vibrational signal lasting 1.0 ± 0.4 s with a fundamental frequency of 330–430 Hz, mainly delivered by pressing the thorax to the comb. The cause of this piping was unclear (Pratt et al., 1996). Like stop signallers that were physically attacked at their food source (Nieh, 2010), these piping workers could remain signalling in the colony for over 1 h (Pratt et al., 1996). Wenner (1964) reported on worker piping (fundamental frequency of 500 Hz, function unknown) that, when transmitted to the comb substrate, elicited freezing, as reported by Michelsen (1986). Recently, Ramsey, Bencsik, and Newton (2017) reported another form of worker piping, a 'whooping signal' that is produced by workers throughout the day and night. This signal may be associated with bees warming the nest since its production rises with decreasing seasonal temperatures and at night.

For worker piping signals, there may be a link between signal reception and response: higher-amplitude vibrations likely elicit stronger sensory neural responses, within limits. Bee subgenual organs, chordotonal organs and campaniform sensilla in the legs and head are sensitive to vibrations (Hrncir et al., 2006; Snodgrass, 1925). Of these organs, more is known about the subgenual organs, which are sensitive to 150–600 Hz vibrations and most responsive at 500 Hz (Kilpinen & Storm, 1997), nicely within the range of worker piping signals such as the stop signal. Whole bee-leg recordings show that the vibrations around 250 Hz should be well detected across three to four comb cells (Sandeman, Tautz, & Lindauer, 1996), although it is unclear how far stop signals can propagate and still elicit responses through the comb. Higher-amplitude signals may transmit farther, although this remains to be determined for worker piping signals, given the complexity of how the substrate constrains signal propagation (Hill & Wessel, 2016).

In general, animals can increase alarm signal amplitude with elevated risk (Blumstein, 2007; Wilson & Evans, 2012). The stop signal, as a referential alarm signal (Tan et al., 2016) fits in well with our understanding of how signal amplitude can convey danger. However, the multiple functions of the stop signal are best encompassed by its role as an inhibitory signal (Nieh, 2010; Seeley et al., 2012). Such inhibitory signalling that actively counters a positive feedback process (Nieh, 2010; Robinson, Jackson, Holcombe, & Ratnieks, 2005) is poorly understood in animal communication. More examples are needed to determine whether heightened inhibition is typically the result of increased signal repetition, signal frequency structure, increased signal amplitude, or a combination of these factors. Based upon our current understanding, such inhibitory signals are also uncommon, although the reason for this rarity is unclear given that inhibitory signalling plays a key role at other levels of biological organization such as in intracellular and neural signalling. The evolutionary implications of this apparent rarity and—given the keystone ecological role played by honey bees—the impact of inhibitory signals upon food web interactions between predators and prey and between pollinators and plant fitness, are fertile grounds for exploration.

Author Contributions

S.H.D., Q.Z. and J.C.N. performed the experiments. J.C.N. and K.T. conceived of and designed the experiments and wrote the manuscript. J.C.N. analysed the data.

Declaration of Interest

We declare that we have no competing interests.

Data Availability

All data are available via Zenodo.org at https://doi.org/10.5281/zenodo.1890984.

Acknowledgments

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