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Colony-level non-associative plasticity of alarm responses in the stingless honey bee, *Tetragonisca angustula*

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Abstract

In ants, bees, and other social Hymenoptera alarm pheromones are widely employed to coordinate colony nest defense. In that context, alarm pheromones elicit innate species-specific defensive behaviors. Therefore, in terms of classical conditioning, an alarm pheromone could act as an unconditioned stimulus (US). Here we test this hypothesis by establishing whether repeated exposure to alarm pheromone in different testing contexts modifies the alarm response. We evaluate colony level alarm responses in the stingless bee, *Tetragonisca angustula*, which has a morphologically distinct guard caste. First, we describe the overall topology of defense behaviors in the presence of an alarm pheromone. Second, we show that repeated, regular exposure to synthetic alarm pheromone reduces different components of the alarm response, and memory of that exposure decays over time. This observed decrease followed by recovery occurs over different time frames and is consistent with behavioral habituation. We further tested whether the alarm pheromone can act as a US to classically condition guards to modify their defense behaviors in the presence of a novel (conditioned) stimulus (CS). We found no consistent changes in the response to the CS. Our study demonstrates the possibility that colony-level alarm responses can be adaptively modified by experience in response to changing environmental threats. Further studies are now needed to reveal the extent of these habituation-like responses in regard to other pheromones, the potential mechanisms that underlie this phenomenon, and the range of adaptive contexts in which they function at the colony level.

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Supplemental Analyses: GLM: We performed a model fit of the data that describes a decay and recovery phase defined by the following equation: $y = c \cdot (1 + (-\text{TauFast} \cdot t) - (\text{TauSlow} \cdot t))$ (c , TauFast , and TauSlow are fit constants, t is time since previous exposure (hrs), and y is one of the colony measures discussed). This gave us a saddle or inflection point for each measure y . We then performed a GLM analysis separately on the decay phase and recovery phase of the model using R (R core team, 2016) looking at the interacting factors of time since previous exposure (t) and total number of alarm pheromone exposures on the measure y . We used a Gaussian distribution or Poisson distribution during these analyses when appropriate based upon the observed data structure of y . We also performed a chi squared model fit test comparing the above model with a null model in which just number of alarm pheromone (AP) exposures was predicting the measure y .

Data Availability

The datasets during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Keywords

stingless honey bee; *Tetragonisca angustula*; colony level plasticity; colony defense; alarm pheromone

Highly social insects, such as honeybees (Apini) and stingless honey bees (Meliponini), build large colonies that provide for brood rearing and storage of resources. Colonies collect resources scattered over the environment and concentrate them in one place for rearing brood and survival though changes in seasons (Wilson 1971; Hansel 1993; McGlynn 2012). Concentrated stores of brood and resources are valuable sources of carbohydrate and protein, which many animals, including other social insects, attempt to exploit. Therefore, a social central-place lifestyle necessitates development of a rapid and coordinated colony defense. Social insects have evolved some of the most sophisticated mechanisms known for communication and coordination of defensive behavior.

In ants, bees, and other social Hymenoptera, alarm pheromones (AP) are widely employed to coordinate nest defense against both conspecifics and heterospecifics (Blum 1969). Alarm pheromones are a common class of pheromones, which elicit defensive and escape behavior in social insects (Maschwitz 1964; Blum 1969). The nature of the response in any individual depends upon its caste and the context in which the alarm pheromone is presented (Maschwitz 1966; Shorey 1973; Sasaki et al. 2014). At or near the colony, alarm pheromones elicit defensive behaviors, especially among specialized guard castes (Wilson 1965; Maschwitz 1966; Blum 1969; Sasaki et al. 2014). Classically, honeybees sacrifice themselves in colony defense against mammals through stinging and subsequently eviscerating themselves to defend the colony (Hermann 1971). A similar sacrificial behavior has been described in several species of stingless honey bees, in which individuals perform a “death-grip” on an object that poses a threat, and those individuals typically stop moving shortly after performing this behavior (Fig S1) (personal observation; Shackleton et al. 2015). In addition to extreme but relatively rare forms of defense, there are more common defensive behaviors that can be more consistently observed across a range of the stingless honey bees. Those include biting, presenting visible guard forces at the nest entrance, application of resins, caustic chemicals and/or toxins to invaders, and reducing the nest entrance size (Butler and Free 1952; Roubik 1989, 2006; Sakagami et al. 1993; Grüter et al. 2016).

When social bees are presented with their species-specific alarm pheromone, they consistently and readily perform defensive behaviors. However, the response incurs a cost via energy expenditure, disruption of foraging activity and potential death it causes to conspecifics. Therefore, it could hypothetically be the case that a truly adaptive response would require behavioral adaptation to match the level of threat. For example, defensive behavior might be reduced in response to repeated low-level threats, such as disturbance via a single non-nestmate scout bee. Alternatively, defensive behavior might be augmented in response to repeated, high-level threats such as a multi-individual raid or attempted raid on the nest in which there is a threat of significant loss of colony resources or workforce. Furthermore, it might be beneficial to learn the pairing of alarm pheromone with the specific

odors of threatening species, such as kairomones, so that the response could be adjusted more reliably in future encounters. Thus the alarm pheromone defense system in social insects might be easily adapted to different situations using non-associative and associative conditioning.

In terms of classical conditioning, a pheromone is an Unconditioned Stimulus (US) (Yunker et al. 1999; Martínez-Ricós et al. 2007). In a motivated animal, a US elicits an innate response in a consistent and stereotypic manner (Makintosh 1985; Rescorla 1988). Typically, the response to the US can be modified by non-associative mechanisms. For example, repeated exposure to a US reduces the response through habituation (Thompson and Spencer 1966; Rescorla 1988). A US can also produce sensitization to other stimuli. Furthermore, a US can be associated with another stimulus – a Conditioned Stimulus (CS) – and modify the response to the CS in a way that is consistent with the pairing (Makintosh 1985).

Here we test hypotheses about colony-level experience-based modification of the alarm pheromone response in the stingless honey bee, *Tetragonisca angustula*, which is common in South and Central America. The nests of *T. angustula* have a single tube-like defense structure with a single nest entrance/exit, patrolled by morphologically distinct guards (Grüter et al 2012; Hammel et al. 2016; Grüter et al. 2017a; Grüter et al. 2017b). These guards perform two types of guarding behaviors, standing and hovering. Standing guards remain on and near the nest entrance tube, and are thought to defend the entrance against conspecific raiders (Kärcher and Ratnieks 2009; van Zweden et al. 2011). Another group of guards constantly hovers in place near the nest entrance, defending primarily against airborne heterospecific raiders (Wittmann 1985; Wittmann et al. 1990; Sakagami et al. 1993; Bowden et al. 1994; Grüter et al. 2011; van Zweden et al. 2011). Using those two populations of guard bees and the forager population, we specifically test the hypothesis that repeated exposure can modify the colony-level response to the alarm pheromone, and that memory of the exposures decays over time. Furthermore, we test whether associations of the *T. angustula* alarm pheromone with the kairomones of other stingless honey bees, which typically attack and raid *T. angustula* colonies, can modify the colony-level responses exhibited by *T. angustula* colonies to kairomones. Such a result would be consistent with associative conditioning and possibly be a first mechanistic step to establishing consistent neighbor relationships in dense social insect environments.

Methods

General experimental procedure

Tetragonisca angustula colonies were observed using a dual camera setup in Gamboa, Colón, Republic of Panama from April to August of 2013. The two cameras were set up with one as a zoom on the *T. angustula* nest entrance and the second as a wide view to capture a 1m radius around the nest entrance. All olfactory stimuli were presented near the nest entrance on a small circular disk of filter paper with a 7 mm diameter pinned to a black sponge (Smith and Roubik 1983). The sponge was used as a visual target, the filter paper and chemicals were not in direct contact with the sponges. Black color has previously been shown to facilitate high levels of defensive response in *T. angustula* (Bowden et al. 1994).

Most interactions were with the filter paper, which was newly prepared for each presentation. As an additional control, after each use sponges were hand washed 4–5 times using an odorless laundry detergent. They were then rinsed with tap water for several hours (20–40 rinses), at which point the odor was no longer detectable. We performed a Pearson correlation analysis to test if this washing procedure was sufficient and found no effect over time on response rates to reuse of sponge controls.

We had access to a total of 16 *T. angustula* colonies over the course of all experiments; 6 colonies kept in man-made hive boxes, and 10 naturally nesting colonies of similar appropriate size and accessibility. Some of the colonies were only used in one experiment, however some were used in multiple experiments. In the latter case, we waited over 30 days between experiments on any colony. According to Grosso and Bego (2002), the adult life span of worker *T. angustula* is near 21 days, making it unlikely that any individuals in a colony experienced more than one experimental treatment. Thus all bees were likely naïve to stimuli upon first exposure. We performed non-destructive tests and therefore do not know the exact size of each colony. However all colonies compared in our analyses were well established and maintained a constant worker output and very similar numbers in both hovering and standing guards at the nest entrance when undisturbed. All of these tests are transient behavioral responses generally lasting less than 15 minutes. During raid events colonies will remain in a highly defensive state for multiple days (personal observation). Thus we are only testing changes in sensitivity to these stimuli rather than a more taxing whole colony mobilization.

All colonies were observed for 2–3 days prior to any experiment to establish baseline activity. An experiment began by observing activity for 2–3 mins just prior to each manipulation. Next, a filter paper control disk with only mineral oil was presented on the black sponge for 3 mins prior to all chemical presentation. Treatment stimuli (natural or synthetic alarm pheromone) were then presented on the black sponge and activity was observed for 10 mins. After the treatment stimulation, a 3-min observation without any chemical stimulus was video-recorded, starting approximately 1–2 mins after chemical stimulus observation.

Natural alarm pheromone stimulus preparation and presentation

Six colonies were observed for responses to natural alarm pheromone between May and June 2013 in Gamboa, Panama. Observation sequence as described above. The natural alarm pheromone was presented using a non-nestmate guard bee. The bee was captured in a vial and then its head was crushed onto a filter paper disk, using forceps to release the alarm pheromone from the mandibular glands (Smith and Roubik 1983).

Stimulation with synthetic chemical alarm pheromone

We shifted to a synthetic alarm pheromone to ensure observed results were specifically a response to alarm pheromone stimulation rather than to other possible compounds released from crushed heads. The synthetic alarm pheromone mixture was diluted to 1% chemical by volume in mineral oil. The synthetic alarm pheromone was composed of: citral (Sigma Aldrich, B1334), 6-methyl-5-hepten-2-one (Sigma Aldrich, M48805), and benzaldehyde

(Sigma Aldrich B1334). Benzaldehyde has been identified in chemical analyses and behavioral studies as part of the alarm pheromone of *T. angustula* (see Wittmann et al. 1990 for discussion of *T. angustula* alarm profile). The latter two are components of the mandibular gland secretion of the robber stingless bee *Lestrimelitta limao*, and therefore kairomones, which also behaviorally elicit defensive behaviors in *T. angustula* (Wittmann et al. 1990; Francke et al. 2000). All chemicals were stored in a refrigerator at approximately 4°C when not in use, and fresh chemical solutions were prepared from a stock solution each week.

Nine previously un-manipulated, completely naïve colonies were exposed to alarm pheromone 6–7 times over the course of 7 days (Fig. 1A) between June and July 2013 in Gamboa, Republic of Panama. Colonies were observed for two days prior to the start of experiments. Then the colonies were tested using the general procedure described above. Colonies were only tested during dry (no rain) conditions and with low wind, because bees generally reduced their activity during adverse weather. Following the pre-experimental observation and mineral oil presentation steps, colonies were exposed to a 1% synthetic chemical alarm pheromone mixture (1% Citral, 1% 6-methy-5-hepten-2-one, and 1% benzaldehyde, v/v) in mineral oil and videotaped for 10 mins. On the first day of experimentation, five colonies were exposed to testing for the first time in the morning and four colonies were exposed to testing for the first time in the early afternoon. On the first day of alarm pheromone stimulation colonies were tested 3 times. Two sets of colonies were established. The testing times for the second set was shifted by approximately 2–3 hours later than the first as a control for circadian changes in defensive behavior. Both sets of colonies were then exposed for the second time approximately 1 hr after the first exposure and then the third time approximately 2 hrs later in the mid- or late-afternoon. The colonies were tested again over subsequent days with increasing time intervals between stimulations approximately 14, 38, 86 and 158 hrs later. In sum, these intervals correspond to approximately 1 hr, 2 hrs, 12 hrs, 24 hrs, 48, and >48 hrs since previous exposure (see Fig 1A for diagram of experimental timeline). Video observations were collected on 56 different occasions, 32 of which were associated with chemical alarm pheromone exposure and 24 of which were prior to experimental onset. Thus we collected a total of 336–560 min (not all pre exposure videos were fully quantified as we collected data for 3 days before and after all treatments) of observational video without chemical alarm pheromone and approximately 310 min of experimental video with chemical alarm pheromone.

Classical conditioning to a novel odor association with alarm pheromone

Five colonies were tested using the colony-level responses to the association of a novel odor with synthetic alarm pheromone (Fig 1B) between July and August 2013 in Gamboa. Colonies were presented with alarm pheromone and odor pairings once every 24 hrs to avoid sensory adaptation or habituation. We used two novel compounds: 3-heptanol (Sigma Aldrich, W354708) and N-octane (Sigma Aldrich, 412236). Novel odor stimuli were diluted to 10% by volume in mineral oil. Three of the colonies experienced 3-heptanol paired with alarm pheromone and the other two experienced N-octane paired with alarm pheromone. For each type of colony, the remaining odor served as an unpaired novel odor comparison (see below).

We defined two different types of experimental days: conditioning days (C) and Test days (T) (Fig 1B). During conditioning days, colonies only had presentations of mineral oil alone (3 min) followed by the pairing of alarm pheromone and the paired odor (either 3-heptanol or n-octane; Fig 1B). Test days occurred on the 1st day, the 5th day, and every subsequent 5th day afterwards throughout the experiment, while conditioning days occurred during the 4 days in between tests. On test days colonies were sequentially presented with the following stimulus sequence and timing (Fig 1B): mineral oil (3 min); the *unpaired* odor alone (10 min); the alarm pheromone-*paired* odor alone (10 min); and then the latter odor and alarm pheromone presented together (10 min). There was an interval of 3 mins between each stimulus presentation. Test days were used to compare responses to the *paired* odor and the *unpaired* odor starting before conditioning and then periodically after conditioning days. All colonies were presented with both odors as a control against changes in behavior due to repetition and not to the pairing procedure. The odors were presented in a non-random order, as we were actively conditioning the colonies on all days and sought to avoid inhibitory effects due to the novel odor not reliably predicting alarm pheromone once every 5 days. Responses on test days were videotaped for offline analysis of behavioral changes to odors. Each colony was measured over a period of 20 days, for a total of 5 test days.

Video Analyses

Video data collection was collected blind to experiment and experiment start date. However, observers were aware of stimulus presented in each video so that the odor identity could be audibly recorded in the video. We defined several parameters related to colony behavior, which we could quantify in videotape playbacks: rate of bees moving into and out of the entrance; number of landing attacks on the sponge visual target; and a general colony activity measure (see Table 1 for full descriptions). We used these parameters to quantify colony-level behaviors. Videos were analyzed using hand counts with an observer over 3-min windows. All videos allowed for identification and counts of foragers exiting and entering the nest as well as the number of bees that attacked the stimulus (Table 1). Numbers of both standing and hovering guards were visually counted at the time of recording, and these numbers were later re-confirmed using wide-view video recordings. All videos were analyzed twice by at least two different individuals to confirm counts were accurate and consistent.

We assessed overall colony activity using SwarmSight software (Birgiolas et al. 2016; Table 1). SwarmSight analyzes pixel changes between frames over a set time. We used SwarmSight to simultaneously quantify pixel changes during the mineral oil presentation and the alarm stimulus presentation. These bouts were filmed within 10 mins of each other from the same angle and distance, and we only collected these data when wind and other factors did not affect observed movement (see Birgiolas et al. 2016 for further information). Thus we could use the mineral oil as a baseline control to quantify potential activity changes in the responses to alarm pheromone treatments. We began analysis 30 seconds after stimuli were presented and quantified pixel changes for 1 min. Hereafter, all mention of activity refers to the above method of pixel change quantification and analysis.

Statistical Analyses

All statistical analyses and plots were made with the R statistics package (R Core Team 2016). We calculated the colony rates for all forager movements, counts for guard behaviors, and overall colony activity, by subtracting mineral oil from activity during alarm stimulation (Table 1). These behavior changes were then compared over time using repeated measures ANOVA, while significant stimuli/observation differences were determined using the Tukey HSD test. All ANOVA tests used colony as a factor, to ensure we accounted for any colony level differences. Time binning for repetitive exposure experiments was determined by the time since previous alarm stimulation. This was done by calculating the time since previous alarm stimulation and adding a binning factor in R using the following categories: first alarm pheromone exposure, less than 2 hrs, 2–12 hrs, 12–24 hrs, 24–48 hrs, and greater than 48 hrs since previous alarm pheromone exposure (Fig 1A). The large windows were due to differences in the onset of first exposure (as stated above some colonies began stimulation in the morning and others began in the afternoon to control for any circadian effects). In order to test if circadian patterns may have an impact on our data, we ran a separate set of ANOVA tests in which we only considered time of day, either morning or afternoon, and colony as factors. We considered a measurement during the morning if observations were made before 12:00 PM, and all observations made after 12:00 PM were considered as afternoon. Additionally, we also performed a GLM analysis and model fitting tests of the same data, which can be found in the supplemental section. These analyses showed the same trends as the presented ANOVA tests (Figure S5).

Results

Topology of the defensive response to alarm pheromone

Tetragonisca angustula colonies differed in forager activity and number of guards. However, the visual target used for the assays did not affect any behavioral measures. There were no differences in forager movement rates caused by the presence of the target, as measured prior to and during presentations (mean $\pm$ SE for: *rate in* target 16.974 ± 2.848 , no target 17.595 ± 2.265 , ANOVA, F-value=0.029, df=1, p=0.866; *rate out* target 15.128 ± 2.987 , no target 12.366 ± 2.062 , ANOVA, F-value=0.579, df=1, p=0.454; *net influx* target 1.846 ± 1.478 , no target 5.228 ± 1.819 , ANOVA, F-value=2.081, df=1, p=0.162). Furthermore, we never observed attacks (Table 1; Fig S1A) on the target in the absence of synthetic or natural alarm pheromone (AP).

Forager Movement: There was no effect of stimulus on the rate of bees entering the nest across treatments (ANOVA, F-value=0.815, df=5, p=0.5403, Fig 2A,). However, there was an effect on the rate of bees exiting the nest (ANOVA, F-value=2.397, df=5, p=0.038, Fig 2B), which decreased during synthetic AP stimulation compared to the period just prior to synthetic AP presentation (Tukey HSD, p=0.019, Fig 2A, b/c). Because of the relative change in these two measures, the net influx rate also increased during synthetic AP presentation, relative to presentations of the sponge visual target alone, mineral oil, and just before synthetic AP (ANOVA, F-value=3.657, df=5, p=0.003; d/ e Tukey HSD, SO/AP: p=0.039, MO/AP: p=0.014, Pre-AP/AP: p=0.006, Fig 2C).

The same general trend of fewer bees leaving the nest was observed during natural AP simulation, causing the net inflow of bees to be positive. However, the differences were not significant (Fig 2D rate in: a, ANOVA, F-value= 0.13, df=2, p=0.878; Fig 2E rate out: b, ANOVA, F-value= 0.154, df=2, p=0.857; Fig 2F net influx: c, ANOVA, F-value= 0.695, df=2, p=0.502).

Guard Movement: During the presentation of synthetic AP there was a decrease in the number of standing guards (standing: ANOVA, F-value=8.314, df=5, p<0.001, Tukey HSD, p<0.001; Fig 3A, a/b), which persisted for several minutes after the removal of synthetic AP (Tukey HSD, p=0.053; Fig 3A, a/b+). However, the decrease was not significant for hovering guards (ANOVA, F-value=0.478, df=5, p=0.792; Fig, 3B). The same general trends were found for the natural AP, but changes were only significant for hovering guards (Fig 3D, ANOVA, F-value=15.30, df=2, p<0.001; Tukey HSD, p<0.001; Fig 3D, b/c) and not standing guards (ANOVA, F-value= 1.546, df=2, p= 0.219; Fig 3C) (Fig 3C,D). The differences in behavior toward natural and synthetic AP presentations could have been due to odor intensity differences between a single crushed *T. angustula* head and the 1% AP mixture used for synthetic stimulation. In addition, other stimuli may have been present in the crushed head.

There was a significant change in flight behavior in the presence of both synthetic and natural AP stimuli. The bees, presumably hovering guards, would perform an erratic flight pattern in which they would quickly fly back and forth in large loops (approximately 12 cm in each direction) within an approximately 0.5 meter radius around the nest. This was apparent in an increase in the overall activity and flight velocity of bees around the nest (See supplementary video).

We quantified the overall activity of colonies during different stimulus presentations by tracking frame-to-frame pixel changes (see Methods; Birgiolas et al. 2016). There was significantly more activity during AP stimulation compared to mineral oil only (MO) stimulation for 5 of the 6 initial AP exposures (paired t-tests, p<0.001, 6th at p=0.19). AP activity was also significantly higher for 27 of 28 paired observations when including all observations irrespective of stimulation timing (paired t-test, p<0.001).

Based upon the above results, we felt the responses to synthetic and natural AP are similar enough to justify exclusive use of synthetic AP for further experimental manipulations. Use of synthetic AP controls for the consistency of stimulation intensity and also for other potentially confounding volatile chemicals that may be present in the heads of *T. angustula* guards.

Non-associative plasticity of alarm responses

We binned our data by elapsed time since previous AP exposure in order to observe the effects of repeated alarm stimulation on colony defensive behavior of *T. angustula*. We found evidence for a habituation-like effect on both guard and forager behavior. This change in behavior was not an effect of circadian rhythms, because there was no effect of time of day on the forager movement rates or colony activity within the windows we observed.

However guard numbers varied throughout the day as previously described by Grüter et al. (2011) (Fig S3).

First we analyzed attacks on either the sponge or the filter paper containing AP using the two measures described in Table 1. There was a significant effect of stimulation interval (SI) on the number of bees attacking and a non-significant effect on the number of attacks counted (Fig 4A, number of bees attacking: ANOVA, F -value=8.097, df =5, p =0.002, Fig S5A, Fig 4B number of attacks: ANOVA, F -value=2.766, df =5, p =0.08; Fig S5B). By both measures, defensive behavior was reduced for AP stimulation 2 hrs after presentation (Tukey HSDs; Fig 4A number of bees attacking: first vs. <2 hrs, p =0.009; Fig 4B number of attacks: first vs. <2hrs, p =0.055). The reduction persisted for up to 12 hrs (Tukey HSDs, first vs. 2–12 hrs, p =0.039; <2 vs. 24–48 hrs, p =0.191; <2 vs. >48 hrs, p = 0.089; Fig 4A). Defensive behavior returned to initial levels at 12 or more hrs after exposure (Tukey HSDs, first vs. 2–12 hrs, p =0.246; Fig 4A, B, Fig S5A, B).

We next analyzed the impact of the time since AP stimulation on the net rate of foraging activity, in the same manner as above, during both AP and mineral oil stimulation (Fig 5, Fig S5C). We found a significant effect of the time since previous exposure on movement rate both during AP presentation (Fig 5A rate in: ANOVA, F -value=8.249, df =5, p =0.003, Fig FB rate out: ANOVA, F -value=8.887, df =5, p =0.002, Fig 5C net influx: ANOVA, F -value=8.249, df =5, p =0.002; Fig S5C) and during mineral oil presentation (Fig 5D rate in: ANOVA, F -value=5.362, df =5, p =0.012; Fig 5E rate out: ANOVA, F -value=3.496, df =5, p =0.043; Fig 5F net influx rate: ANOVA, F -value= 6.839, df =5, p =0.005). Foragers displayed reduced rates of nest entry 2–12 hrs after AP exposure but not at the shortest stimulation interval during both AP (Tukey HSD, rate in: first vs. 2–12 hr, p =0.001, <2h vs. 2–12 hr, p =0.003; Fig 5A-C, Fig S5C) and mineral oil stimulation (Tukey HSD, first vs. 2–12 hr, p =0.009; <2 vs 2–12 hr, p =0.062; Fig 5D-F).

During AP stimulation, colonies also showed decreased rates of bees exiting the nest 2–12 hrs after previous exposure (Tukey HSD, rate out: first vs. 2–12 hr, p =0.03, <2 vs. 2–12 hr, p =0.017; Fig 5B). This corresponds to the first experiment presented (Fig 1A-B), in which the rate of bees exiting seemed to be most affected by the presence of AP (Fig 2B). Interestingly, during mineral oil stimulation bees also showed reduced exit rates compared with longer SI times (Tukey HSD, 2–12 hr vs. 12–24 hr, p =0.067; 2–12 hr vs. 24–48 hr, p =0.049; Fig 5E), but not between the first exposure and subsequent rates during the shortest interval.

When we measure the net forager activity during AP stimulation, the net influx rate began positive; then with repeated AP stimulation it reduced to a value similar to that observed during mineral oil stimulation. The next influx rate became closer to a zero value but did not fully recover within the shorter stimulus intervals (first vs. 2–12 hr, p =0.007, <2 vs. 2–12 hr, p =0.017; Fig 5C). During mineral oil exposure we also observed differences in net influx rates (Fig 5F). Strangely, the net influx rate was significantly more reduced at stimulus intervals greater than 12 hrs (Tukey HSDs, first vs. 12–24 hrs, p =0.004; <2 vs. 12–24 hrs, p =0.018, 2–12 vs. 12–24 hrs, p =0.018; Fig 5F) and normalized at stimulus intervals greater than 48 hrs. Given the previous observation that mineral oil and the presence of visual

targets did not have an effect on forager activity, this suggests a more long-term alteration in forager activity after several AP stimulations.

Last, we looked at the impact of the rate of AP stimulation on overall activity. We found there was an effect that approached but did not reach statistical significance of repeated AP stimulation on the overall activity at the nest entrance (ANOVA F-value=10.47, df=5, p=0.089; Fig 6, Fig S5D).

Associative alarm responses

We also attempted to pair AP as an unconditioned stimulus with two different non-pheromone/kairomone odor conditioned stimuli (CSs). We compared the first response to the CS with subsequent responses after several CS-AP(US) pairings. There was no impact of experience (CS-US pairings) on response to the CS for the net influx rate (ANOVA, F-value=2.332, df=1, p=0.129; Fig S4). Guard measures and nest activity measurements were also not significantly different (Table S1).

Discussion

Adaptive topology of colony defense behavior

The defensive response of *Tetragonisca angustula* involves both ‘standing’ and ‘hovering’ guards (Wittmann 1985; Grüter et al. 2011; Güter et al. 2017a). The former take up positions on the nest entrance tube either just inside the entrance or around the outside of the tube near the entrance. Hovering guards fly in a fairly stable position relative to the nest entrance tube and are oriented toward the entrance (Ziel and Wittmann 1989; Kelber and Ziel 1990). We find that during presentation of synthetic or natural alarm pheromone the number of hovering guards increases. The increase most likely results from the recruitment of the standing guards, because the number of outbound departures from the nest entrance is reduced. However, the increase in total hovering guards is on average greater than the number reduced from the standing pool (Fig S2). This would suggest that there was recruitment of guards from inside the nest or incorporation of returning foraging bees as exterior nest guards. Furthermore, we find that the colony-level defensive response changes with experience, and these changes are most consistent with habituation.

One of the main threats to any stingless honey bee colony comes from other meliponine bee species that raid colonies (Sakagami et al. 1993; Cunningham et al. 2014; von Zuben et al. 2016; Grüter et al. 2017b). In the context of a non-nestmate or heterospecific scout discovering the nest entrance, the goals of a colony should be as follows. Outside the nest, guards need to quickly immobilize the threat and minimize loss. In order to accomplish this, guards may actively search for and eliminate foreign (non-nestmate and heterospecific) scouts outside the nest. Inside the nest, nestmates should maintain the integrity of colony brood and other critical resources in the event that any raiders gain entry.

The responses we observed during alarm stimulus presentations are consistent with this interpretation. The colony increases the number of actively hovering guards. At the same time, outgoing activity is reduced, which increases the number of bees within the nest who could defend the colony and the queen if intruders breach the perimeter. Other reports have

described that, in the event of a raid and loss of the nest entrance integrity, nestmates stand over brood and other critical resources to reduce the potential impact a raid event has on the colony survival (Sakagami et al. 1993).

Qualitatively the responses to both the natural and synthetic alarm pheromone stimuli are very similar. However, there were clear quantitative differences between the natural and synthetic alarm pheromone stimuli response levels. These differences were most likely due to two possibilities. First there could be quantitative differences in alarm substance released between a single crushed *T. angustula* head and the 1% AP mixture we used for synthetic stimulation. Second, there may have been other volatiles in the bee head, which have not been completely quantified or tested in *Tetragonisca angustula*. It is clear from the work of Wittmann et al. (1990) that the responses of *T. angustula* to their alarm components are robust to chemical composition.

The responses we see to both natural and synthetic alarm pheromone stimulation are consistent with those observed in prior *T. angustula* studies as well as other studies involving social bees. A recent study by Gong et al. (2017) has shown reduced forager activity in response to the primary component of the honeybee alarm pheromone, isopentyl acetate, in both the Asian and European honey bees. This is consistent with what we observe here with *T. angustula* foragers. Additionally, reduced forager responsiveness has also been demonstrated in the lab using harness bees, which perform very poorly in proboscis extension tasks in the presence of alarm substance (Urlacher et al. 2010). Thus the natural and synthetic alarm pheromone responses we observe in *T. angustula* appear to be robust across social bees. However, the unique guard behaviors of *T. angustula* provide additional insight into the whole colony response, which is not possible to easily observe in other bee species.

Non-associative modification of the defense response

The colony-level defense behaviors of *T. angustula* are modified by experience in ways that are consistent with habituation. Habituation is an adaptive behavioral phenomenon through which the response to a stimulus decreases with repeated exposure (Harris 1943; Thompson and Spencer 1966; Rescorla 1988, Rankin et al. 2009). Thomson and Spencer (1966) describe several parametric characteristics of behavioral habituation, later revised by Rankin et al. (2009), which can be used to dissociate it from sensory adaptation. We report two critical points for demonstrating habituation. The first criterion is a decrease in behavioral response with repeated stimulation. We find a decrease in guard attacks, forager movement, and activity in response to repeated alarm pheromone stimulation. Second, we observe spontaneous recovery to approximately normal baseline defensive responses with intervals of 24 to 48 hrs.

Our experiments cannot fully eliminate sensory adaptation, which refers to a decline in response of olfactory sensory cells that detect a stimulus, such as alarm pheromone. Adaptation is well known to occur in olfactory receptor neurons, and brief exposure to an odor produces both a decline in responsiveness of the sensory neuron as well as a decline in behavioral response to the odor (Kaissling et al. 1987; Colbert and Bargmann 1995; Kurahashi and Menini 1997). However, because of the time frames involved in our tests,

sensory adaptation is less likely. The longest lasting form of sensory adaptation affects sensory cells on the order of at most a few hrs (Colbert and Bargmann 1995; Zufall and Leinders-Zufall 2000). Reported sensory adaptation durations vary by animal: salamanders 6.5 mins (Zufall et al. 2000); the fruit fly for 1.5 min (Störtkul et al. 1999); house fly for 15 mins (Kelling et al. 2002); the worm *C. elegans* for approximately 3 hrs (Colbert and Bargmann 1995); silkworm moth for approximately 1 hr in isolated antennae (Kaissling et al. 1987); the rabbit for 30–50 min when adapted for 1 hr (Chaput and Panhuber 1982). In our study we find reduced behavioral responses to olfactory alarm stimulation lasting up to 12 hrs, which would be unlikely to be due to sensory adaptation.

Therefore, reductions in behavior that we report could be due to habituation, which can last at least for days as it progresses through different forms of memory consolidation (Tully et al. 1994; Menzel 1999). In honeybees long-term appetitive habituation has been shown to last at least 24 hrs (Bicker and Hähnel 1994). We did not, however, have the opportunity to test additional behavioral characteristics that affect habituation, such as: stimulus strength; generalization of habituation to other stimuli; or dishabituation. The last characteristic refers to re-establishment of the behavioral response just after presentation of a strong sensitizing stimulus, such as disruption or a disturbance of the colony, or perhaps presentation of the alarm pheromone of a parasitic species of stingless honey bee (e.g. *Lestrimelita*; Sakagami et al. 1993). Demonstration of dishabituation, coupled with electrophysiological measurements from the sensory cells on the antennae, would be the next essential steps in testing the habituation hypothesis for *T. angustula*.

Circadian rhythm cannot account for the reduction in behavior that we observed. Five colonies were initiated in the morning and four others were initiated several hours later. As a result, the alarm behaviors as a function of initial treatments were tested at different times of day. Furthermore, the behaviors that change as a result of experimental manipulation did not show differences between the two onset times. We observed circadian patterns in guard number as had previously been shown (Grüter et al. 2011), but that would not account for the reductions we report.

At first it may seem puzzling that a colony would reduce alarm responses to repeated alarm pheromone delivery. A colony needs to be able to maintain the defense of resources especially in the face of repeated attack. The decline in responsiveness we report could allow a colony to ‘tune’ the alarm response in a way that is adaptive to different levels of regularly occurring threats. A low level of threat might consist of an occasional scout bee from a potential raiding colony. This situation could be analogous to the condition that we presented to the colonies. Bees generally did not die during alarm presentations (with the exception of the few bees that performed a ‘death grip’ on the AP filter paper), and there were no large-scale raids from non-nestmate bees. Thus there was no immediate threat to the colony. In this case, the adaptive response to repeated low-level threats would be to conserve energy and resources by reducing the response to a mild threat. Accordingly, Tan et. al (2016) have shown colony-level response tuning to wasps of varying threat levels in the Asian honey bee, *Apis cerana*.

We also report that different behaviors show habituation to AP in different time frames. These behaviors represent different behavioral castes within the stingless honey bee colony (Hammel et al. 2016). Attacking guards habituate most rapidly, followed by foragers at between 2–12 hrs, and then general activity reduces during from 12 to 48 hrs after stimulation. These responses provide an adaptive response that could reduce disruption of foraging activity and minimize guard energy expenditure, while still balancing the ‘vigilance’ of the colony response to a threat.

Long term responses to repeated alarm pheromone stimulation

Colony responses, including both forager activity and guard behavior, adapt to repeated alarm stimulation for extended periods of time. Early comparisons establish that the presence of mineral oil and the visual target do not have an impact on forager activity. Yet, we see changes in forager activity after repeated alarm pheromone (AP) stimulation during the mineral oil observation period (Fig 5D-F). This suggests that repeated alarm pheromone stimulation has impacts on foraging activity for at least for 2 to 24 hrs after the window of AP observation. Moreover, the reduction generalizes to the tests with mineral oil, indicating that it is a broad, colony-wide response. We also observe long term changes in guard behavior but they only manifest during alarm presence. The altered response profiles for both foragers and guards are long lasting (2–48 hrs), but manifested in different ways. Guards only attack or demonstrate activity during AP stimulation, and therefore only demonstrate altered response profiles during AP stimulation (Fig 4). Foragers on the other hand are always active, and thus manifest altered activity with and without AP stimulation for 2–12 hrs after AP stimulation but without alarm pheromone present (Fig 5).

Associative conditioning with alarm pheromone

We found no evidence that colonies of *T. angustula* modified their behavior in response to novel odors paired with synthetic AP. Thus we cannot at present conclude that the association of an AP with kairomones of other bees can modify the colony response to the kairomone. However, we occasionally observed individuals performing ‘death grip’ behaviors on filter paper with novel odor in addition to the filter paper containing AP. The most parsimonious explanation is that the bees stimulated by the AP were simply performing the ‘death grip’ on any novel stimulus in the vicinity of the AP stimulus. Or the biting behavior could be directed at any odor near the AP. It remains possible, however, that the bees could have learned the association of AP with the novel odor and performed the ‘death grip’ in response to the novel odor, just as they would to the AP. Recent work by Dawson et al. (2016) reports individual associative learning using alarm pheromone paired with colored light in the honeybee, *Apis mellifera*. So it is possible that conditioning at the individual-level occurred, but we were unable to capture this with our colony-level measures.

Group versus individual conditioning

All of our experiments involved treating groups of individuals. We therefore report evidence of adaptive group-level modification of behavior. This could be acquired in two ways. First, it could be acquired through individual experience. Second, it could be acquired through indirect experience via colony communication or modification of some colony level signal.

We assume that a decline in response when tested several hrs later reflects, in some way, individual learning via one or both means. However, we did not track individuals in our experiments, which would have been difficult because of the situation of the colonies and because of the size of the bees. Therefore, we have no way of knowing if the individuals present during the test were the same ones present during the treatment. It therefore remains an open, interesting question how individuals learned in our experiments. According to Grüter et al. (2011), individual standing guards perform guarding behavior approximately 1–2 hrs at a time, and hovering guards perform guarding behavior for about 1 hr at a time in *T. angustula*. It is not currently known how frequently a *T. angustula* guard performs guarding behavior. Individual honeybees (*Apis mellifera*) perform both guard and foraging tasks with some circadian regularity (Moore et al. 1998, Troen et al. 2008), thus it is possible that *T. angustula* switches between these activities. However, given that the reduction of attacking behavior and foraging activity extends beyond the 2 hr window of initial testing, it seems likely that different individual bees were involved between initial exposure and testing at different times periods up to 12 hrs later. If the individuals differ during a test, then it remains an open question how they learned about the alarm pheromone exposure without having performed the behavior during the initial treatment. In conclusion, our experiments raise the important issue of how individual and colony factors come into play to disseminate learning in groups to shape colony-level defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

Pheromones are classically thought to elicit stereotyped action patterns. Here we test the idea that responses to pheromones are plastic and show characteristics of an unconditioned stimulus. This study demonstrates clear non-associative plasticity in the colony level response to alarm pheromone, in the stingless honey bee, *Tetragonisca angustula*. Colonies of *T. angustula* show habituation-like responses across multiple measures to repeated stimulation of their alarm pheromone. We therefore demonstrate that colony-level responses to pheromones are adaptively plastic. Finally, we failed to demonstrate colony-level conditioning using alarm pheromone as the unconditioned stimulus, however these findings and others warrant further investigation.

Figure 1.

A. Time line for stimulus presentation during stimulation with synthetic alarm pheromone. The above vertical lines along the time line represent times at which a colony was presented with alarm stimuli. During each alarm stimulation (vertical line), we presented and collected data in the following sequence: first with visual target-sponge only, second mineral oil on visual target, third 1% synthetic alarm pheromone mixture (AP), and last a post observation of the visual target alone. The bottom vertical lines represent continuous time in hours of the experiment. **B.** Time line for classical conditioning to a novel odor alarm association experiment. The vertical lines along the time line represent stimulus presentation. There were two types of stimulus presentation, conditioning days (C) and test days (T). On conditioning days colonies were presented with stimuli in the following order: sponge visual target only, then mineral oil on visual target, and last the 1% synthetic alarm pheromone (AP) and 10% transfer odor in mineral oil on two separate filter paper pieces. On test days, stimuli were presented separately and then paired to determine if there is altered behavior to novel transfer stimuli alone. We presented stimuli in the following order on t days: sponge visual target only, mineral oil on visual target, non-AP-associated novel odor on visual target, AP-associated novel odor on visual target, and finally the paired AP and AP-associated novel odor together on visual target.

Figure 2.

The mean movement rates of foragers in bees per min during various stimulus presentations pooled across the synthetic alarm pheromone experiment (A-C). The rate of bees entering the nest (A, dark gray); the rate of bees exiting (B, gray); and overall net flux of bees entering the nest (C, light gray). Error bars represent standard error of the mean. Treatments are: SO (sponge visual target only), MO (mineral oil on sponge), AP (synthetic alarm pheromone on sponge), Pre- and Post- AP (sponge only before and after synthetic alarm pheromone presentation). Letters denote significant differences: (a) denotes rate in comparisons, (b) and (c) denote rate out comparisons, and (d) and (e) denote net influx comparisons. (Tukey HSD * $p < 0.05$). The mean movement rate of foragers (bees per min) before, during and after presentation of natural alarm pheromone (D-F). The rate of bees

entering the nest (**D**, dark gray); the rate of bees exiting (**E**, gray); and overall net flux of bees entering the nest (**F**, light gray). Error bars represent standard error of the mean. Treatments are as follows: NP (crushed bee head), Pre- and Post- NP (sponge only before and after natural alarm pheromone presentation). A total of 342 observations of 9 colonies and 5 types of stimulation for synthetic AP and 100 observations of 9 colonies and 3 types of stimulation for NP.

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Figure 3.

The mean number of guards before (Pre-AP), during and after (Post-AP) synthetic alarm pheromone presentation (AP). Standing guards (**A**, dark bars) and hovering guards (**B**, lighter bars). Error bars represent standard error of the mean. Letters denote significant differences: (**a**) and (**b**) show standing guard comparisons, and (**c**) shows hovering guard comparisons. (Tukey HSD *** $p < 0.001$, + non-significant, $p = 0.053$) The mean number of guards before (Pre-NP), after (Post-NP), and during natural alarm pheromone presentation (NP). Standing guards (**C**, dark bars) and hovering guards (**D**, lighter bars). Error bars

represent standard error from the mean. Letters denote significant differences: (a) denotes standing guard comparisons, (b) and (c) denote hovering guard comparisons (Tukey HSD *** $p < 0.001$). A total of 342 observations of 9 colonies and 5 types of stimulation for synthetic AP and 100 observations of 9 colonies and 3 types of stimulation for NP.

Figure 4.

The mean number of attacks and estimate of bees attacking during synthetic alarm pheromone presentations binned by time since previous exposure to synthetic alarm pheromone. The number of bees attacking (**A**, dark gray), and the number of attacks on synthetic alarm pheromone stimulus (**B**, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denotes number of bee estimate comparisons, and (c) denotes number of attack comparisons (Tukey HSD *p<0.05, **p<0.01, + non-significant p= 0.055). A summary of 39 alarm presentation observations of 6 colonies.

Figure 5.

A-C. The mean forager movement rate (bees/min) during synthetic alarm pheromone presentation binned by time since previous exposure to synthetic alarm pheromone. Rate of bees entering the nest (**A**, rate in, dark gray), rate of bees exiting the nest (**B**, rate out, medium gray), and net rate of bees entering the nest (**C**, net, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denote rate in comparisons, (d), (c), and (e) denote rate out comparisons, and (f) and (g) denote net influx rate comparisons. (Tukey HSD + non-significant, $0.067 < p < 0.05$) **D-F.** The mean forager movement rate (bees/min) during mineral oil only presentation binned by time since previous exposure to synthetic alarm pheromone. Rate of bees entering the nest (**D**, rate in, dark gray), rate of bees exiting the nest (**E**, rate out, medium gray), and net rate of bees entering the nest (**F**, net, light gray). Error bars represent standard error of the mean. Letters denote significant comparisons: (a) and (b) denote rate in comparisons, (d) and (c) denote rate out comparisons, and (f), (g) and (h) denote net influx rate comparisons. A summary of 39 alarm presentation observations of 6 colonies.

Figure 6.

The mean percent change in flight activity from synthetic alarm pheromone (AP) and mineral oil (MO) presentations (measured by frame-to-frame changes in video pixels) binned by time since previous exposure to AP. Error bars represent standard error from the mean. The letters denote comparisons with (b+) as approaching significance— $p=0.1$ —from (a) and no difference from (ab). A summary of 39 alarm presentation observations of 6 colonies

Table 1.

The list of behavioral terms used and description of behaviors/measurements those terms refer to.

Behavioral Term	Description of Behavior
<i>Attacks</i>	Short range aggressive pursuit behaviors directed toward either sponge or olfactory stimuli. The bees fly in a pattern with head oriented toward the stimulus then a bee raises the legs toward stimulus and quickly fly towards and grip stimulus. This behavior is often, but not always, followed by the bee biting the stimulus with its mandibles. This is a quantification of these observed attacks or aggressive pursuit and grip behaviors.
<i>Number Attacking</i>	This is an estimate of the number of bees that are performing the attacks. This measure was done to ensure that behaviors were truly colony level behaviors and not due to one or a few very stimulated individuals.
<i>Rate in</i>	The rate of bees entering the nest via the nest tube.
<i>Rate out</i>	The rate of bees exiting the nest via the nest tube.
<i>Net Influx rate</i>	The difference between the rate of bees entering the nest and the rate of bees exiting the nest. Thus a positive number indicates more bees are entering than exiting, and a negative number indicates more bees are exiting than entering.
<i>Activity</i>	A measure calculated using the SwarmVision software (Birgiolas et al. 2016) to quantify pixel changes over a one min window within a video. In all cases we used a relative change between paired observations by subtracting the pixel changes during mineral oil stimulation from the pixel changes during synthetic alarm pheromone stimulation. We did this to ensure we account for variation in hive size and overall activity between nests.