



The Night Shift: Nest Closure and Guarding Behaviors in the Stingless Bee, *Tetragonisca angustula*

Meghan M. Bennett · Kaitlin M. Baudier

Received: 29 January 2021 / Revised: 7 July 2021 / Accepted: 12 July 2021

This is a U.S. government work and not under copyright protection in the U.S.; foreign copyright protection may apply 2021

Abstract Social insects face threats to the colony during the day but also at nighttime. Multiple strategies have evolved across social insect species that protect the nest and each other at night. We asked what behaviors are employed by stingless bees, *Tetragonisca angustula* at night. We describe two layers of defense, nightly nest closing and night guarding at the entrance. After monitoring 10 naturally occurring colonies, we saw considerable variation in the likelihood of colonies leaving a nest open or closing them at night. Furthermore, more guards were at the entrances of open nests than closed nests. The mass of nightly guarding bees and tube closers was consistent with (even slightly exceeding) the mass of daytime guards, suggesting that these tasks are performed by especially large soldier bees. We found

preliminary evidence supporting the hypothesis that the kairomone citral, released by obligate kleptoparasites, encourages nest closing behavior. Two nests that were consecutively open for a week, partially closed the night we applied alarm pheromone. These data are just a glimpse into some of the nightly defensive behaviors and what shapes nest closure behavior. We discuss how these data are an important first step and discuss hypotheses emerging from this study.

Keywords Abejas angelitas · circadian · division of labor · group defense · nocturnal defense · soldier

Introduction

Social insects have evolved an impressive array of defensive behaviors to protect their colonies from invasion. Honey bee guards stand at the entrance of the colony and touch incoming bees to identify nest-mates via cuticular profiles (Dani et al. 2005). Some termite soldiers utilize specialized mandibles to create defensive ballistic movements (Seid et al. 2008) or produce defensive secretions from mandibular glands (Prestwich 1979). Certain eusocial bees, ants, and gal aphids are even known to, at times, sacrifice individual colony members in order to protect the group from invaders (Kurosu et al. 2003; Tofilski et al. 2008; Shackleton et al. 2015). The study of collective defense in social insects has not only informed contemporary theory of allocation and specialization

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10905-021-09779-9>.

M. M. Bennett
USDA-ARS Carl Hayden Bee Research Center, 2000 East Allen Road, Tucson, AZ 85719, USA

M. M. Bennett (✉) · K. M. Baudier
School of Life Sciences, Social Insect Research Group,
Arizona State University, Tempe, AZ 85281, USA
e-mail: meghbennett@gmail.com

K. M. Baudier
School of Biological, Environmental and Earth Sciences,
The University of Southern Mississippi, Hattiesburg,
MS 30406, USA

among highly coordinated animal societies (Wilson 1968; Gordon 1996) but has also served as inspiration for biomimetic swarm robotics and cyber protection (Korczynski et al. 2016; Strickland et al. 2018; Kwa et al. 2020). Thus, expanding our understanding of defense strategies of social insects provides insight into evolutionary behavioral trajectories and can foster cross-disciplinary innovation.

Within a eusocial insect nest, the most vulnerable point of intrusion is often the entrance. Colonies must balance group defense with the need for efficient traffic flow among colony members as they perform essential colony functions (e.g. foraging, waste removal, construction). Strategies to protect nest entrances are diverse across social insect species and include both architectural (Jeanne 1975; Roubik 2006) and behavioral defenses (LaPolla et al. 2002; van Zweden et al. 2011). Many species across genera of stingless bees manipulate their nest entrance tubercle, such as *Friesella*, *Meliponula*, *Nannotrigona*, *Pariotrigona*, *Plebeia*, *Scaptotrigona*, *Scaura*, *Schwarziana* and *Trigona* (reviewed in Roubik 2006). Manipulation of the nest entrance may encourage a trade-off between foraging and guarding. For example, in *Paramona helleri* colonies, nest entrance tubercles are mostly sealed with the exception of small holes so foragers can pass through easily, with fewer guards having to defend the opening (Shackleton et al. 2019). Diurnal stingless bee species such as *Tetragonisca angustula* are interesting models in which to study tradeoffs between architectural versus behavioral strategies for nest defense because they perform highly specialized nest guarding behaviors in the day (Wittmann et al. 1990; Bowden et al. 1994; Roubik 2006), but are also believed to rely heavily on physical fortifications of nest entrances for colony defense, especially when activity ebbs at night (Roubik 2006). However, while defense behavior during peak daytime activity in this and other eusocial insect species has been well described, far less is known about how colonies defend their nests at times of diel inactivity. This study delves deeper into nighttime rhythms of *T. angustula* behavioral and architectural nest defense.

Colonies are faced with different threats between day and night. Daytime threats for many tropical bee colonies include intense kleptoparasitization from other bees (Grüter et al. 2016), while nocturnal ants are common nighttime intruders (Roubik 2006). For example,

predominantly nocturnal *Ectatomma* ants have been shown to prey upon colonies of *T. angustula* (Ostwald et al. 2018) and sweat bees, *Lasigoglossum umbripenne* (Schatz and Wcislo 1999). Some species of stingless bees will physically close the nest with batumen of cerumen (wax mixed with plant resin) (Roubik 2006). *Tetragonisca angustula* colonies either close, partially close, or leave open the nest entrance tube (Roubik 1992; Grüter et al. 2011). Even within a single colony, nest closure behavior can vary day to day. However, little is known about what cues mediate nest closure, or which individuals in the colony are performing this behavior.

Within behavioral defense strategies, social insects differ in how individuals are allocated to nest defense. In some termites, ants, and stingless bees, morphologically distinct guards called “soldiers”, notably larger than other sterile workers, behaviorally specialize on defense-related tasks (Miller 1969; Gordon 1996; Grüter et al. 2017). In other instances, like in honey bees, behavioral shifts to guarding among workers is an age-polyethism (Seeley 1982; Page Jr et al. 2006). In the case of *T. angustula*, morphologically larger soldier bees age through one of two diurnal defense tasks in their final two weeks of life (Grüter et al. 2012; Hammel et al. 2016). Younger soldiers hover near the nest entrance (hovering guards) and older soldiers stand at the entrance of the nest (standing guards) (Baudier et al. 2019). During the day, these guards protect the colony and its resources from heterospecific and conspecific robbing by predominantly other stingless bees (Grüter et al. 2016). We ask whether night guards and nightly tube manipulators belong to any of these known morphotypes or age groups. We hypothesized that night guarding, like diurnal guarding, is performed by morphologically larger soldiers. We then ask if night guards perform other defense tasks during the day using paint marking methods. Via these novel investigations into the behavioral, morphological, and architectural nighttime defenses of this tropical bee, we create a more wholistic picture of their dynamic diel defense strategy.

Materials and Methods

Field Site

All fieldwork was performed on naturally occurring nests in and around the town of Gamboa (9.12° N,

79.70° W) in Colón province, Panama. Of these 10 subject colonies, 4 had nested in tree cavities, and 6 were nested within man-made objects (4 cinder block walls, 2 electric meters). All work was performed in February 2019, during the dry season peak of bee foraging activity.

Characterizing Diel Fluctuations in Colony Activity Over 24 Hours

We observed externally visible bee behavior and nest entrance tube conditions of all 10 colonies for 24 h in 2-h increments (checking all nests took approximately 45 min in total). This was done starting at 06:00 on 16 February 2019 and was completed at 04:00 on 17 February 2019. At each time interval, we took a photo of the nest entrance with a reference ruler (accurate to the nearest 1 mm) so that areas of each tube opening could be measured from photos using ImageJ (Schneider et al. 2012). This allowed us to compare the degree of tube closure across colonies and times of day. At each time interval we also recorded the number of bees participating in several focal nest entrance behaviors (Table 1).

We tested for significant circadian patterns on a 24-h clock using circular statistics. Using the R studio package “Circstats” we generated test statistics like circular means and median time of activity for each task group. We tested the hypothesis whether time of activity was uniformly distributed or unimodal using Rayleigh’s test of uniformity. The test statistic r_{bar} ranges from 0 to 1, and the mean resultant vector is uniform when r_{bar} is closer to 0, or unimodal when closer to 1. For data suspected of bimodality, we used

a Hermans-Rasson’s test of non-uniformity as this test is more accurate than the Rayleigh test (Landler et al. 2019). This test was achieved using the R studio “CircMLE” package.

Testing for Inter-Colony Differences in Closure Across Nights

To test whether colonies consistently differed in their tendency to close nest entrance tubes at night, we made observations of natural nest closures for each of the 10 colonies across 6 nights. Each night we observed whether nests were open or closed at 20:30 to 21:30 (“open” in this case being qualified as having at least one hole the size of a bee’s head or larger). This was done February 1 through February 6.

From day 3 through 6 (February 3 through February 6), we also assessed the degree of relative closure via photo analysis. We photographed nest entrance tubes alongside rulers during mid-day (12:00 to 15:00) and in the evenings (20:30 to 21:30). Areas of each tube opening were measured from photos using ImageJ. We used these data to test whether the degree to which colonies closed entrance tubes each night ($\% \text{ closure} = (\text{daytime area} - \text{nighttime area}) / \text{daytime area} * 100$) was consistently different across colonies using a repeated-measures ANOVA.

Identifying Morphological Caste of Night Guards

To test the hypothesis that soldiers (morphologically distinct, larger-bodied workers) perform night guarding and closing tasks, we gathered, weighed, and marked bees seen performing closing and

Table 1 Ethogram describing focal behaviors observed at the nest entrances of *T. angustula* colonies in this study. At night we backlit nest entrance tubes with a flashlight to check for shadows of night guards, closers, and openers. Presented

descriptions of standing guarding, hovering guarding, and foraging at nest entrances are established (Zeil and Wittmann 1989; Kärcher and Ratnieks 2009; Baudier et al. 2019). Descriptions of nest opening and closing as well as night guarding are novel

Behavior	Description
Night guarding	Bees standing motionless on the inside of the nest entrance tube at night while facing the distal end of the tube
Closing nest	Bees chewing on the nest entrance tube during evening hours (16:00–22:00)
Opening nest	Bees chewing on the nest entrance during morning hours (04:00–10:00)
Hovering guarding	Soldiers hovering at the nest entrance flying in static inward-facing flight (> 20 s) formation paired along the flyway
Standing guarding	Soldiers standing motionless (> 20 s) anywhere on the nest entrance tube facing the tube entrance during the day
Foraging	Bees that exited the nest entrance tube and immediately flew away from the nest without carrying waste material

night-guarding behaviors. Colonies were checked for closers and night-guards on two nights following cross-night closure observations (7 & 11 February 2019). These bees were collected, weighed to the nearest tenth of a milligram, paint marked using an oil-based paint pen, and returned to their natal colony. For comparison to known size groups, we collected day-time foragers (minors) as well as standing and hovering guards (soldiers). We collected a total of 15 day-shift comparison bees (5 per task) from each colony where night-shift bees were collected. Guards were only collected after the inter-colony differences data collection was completed so that modification would not affect nightly nest closure. This sampling size was used to approximately match N for collected night-shift bees. Bee mass was compared across task groups using a mixed-model ANOVA (lme4 package in R) which included colony ID as a random factor. A post-hoc Tukey HSD was used for between-task comparisons.

To further test our hypothesis that soldiers perform nest closure and night-guarding activities, we checked colonies daily and nightly for reemergence of paint-marked individuals starting 11 February 2019, with daily diurnal spot-checking of foragers, hovering guards, and standing guards in the two weeks following as we proceeded with other work.

Testing for a Tradeoff Between Tube-Closure and Night-Guard Investment

Stingless bee nests face a variety of nocturnal threats, but soldiers are expensive to produce (Segers et al. 2015). Therefore, we expected a tradeoff between nest closure and night guard number, that colonies tending towards more open nighttime nest entrance tubes would station a higher number of night guards in entrance tubes than those which close fully. To test this hypothesis, we compared the number of night guards at the entrance of open and closed nest entrance tubes (as previously defined) for 6 days using a Welch two sample t-test.

Testing Hypothesized Sources of Night Closure Variation

One potential explanation for why some colonies differ in the extent to which they invest in nightly tube closure versus open-tube high night-guarding

is colony size. Larger colonies have more soldiers (Segers et al. 2016) and greater need to open tubes efficiently for high-traffic use in the day, potentially making tube closure less favorable and night guarding less costly. To test this hypothesis, we conducted two separate linear regressions: 1) maximum colony traffic rate (proxy for colony size, from 24-h observations) versus colony average nightly tube closure % (from multi-day photo measurements) and, 2) maximum colony traffic rate versus colony maximum night guard number (as recorded in 24-h observations) v. Traffic is a commonly used noninvasive method of estimating colony size in this species (Grüter et al. 2011; Segers et al. 2016). Under this hypothesis we predicted a negative correlation for the former, and a positive correlation for the latter.

The volatile chemical citral is an important component of recruitment pheromone in raids of *Lestrimelitta* spp., major kleptoparasites of *T. angustula* (Blum et al. 1970). Citral is also detectable and excitatory to soldiers of *T. angustula* (Balbuena and Farina 2020; Baudier et al. 2020). We tested the hypothesis that inter-colony differences in nightly closure are a plastic consequence of inter-colony differences in kleptoparasite loads. We added 5ul of undiluted citral (Spectrum, Gardena, CA) to a piece of filter paper near nest entrances and observed the effect on nest entrance closure. Colonies that were reliably open or closed sequentially for 6 nights (Fig. 2, bottom panel) were used. CH and PK were two colonies consistently closed. SC and TR were consistently left open at night. Citral was applied near the nest entrance at 20:00 during the peak time of observed nest closure activity (Fig. 1). ImageJ was used to quantify the area (cm²) of the nest openings from photos taken immediately before application of citral, 6 h following citral application (02:00) and to time-matched photos taken at 20:00 and 02:00 from the 24-h study.

Results

Fluctuations in Entrance Closure and Colony Activity Over 24 Hours

Over 50% of nests were open to some degree at 06:00 (Supplementary figures S1 & S2), though all colonies increased nest entrance sizes further between 06:00 and 08:00. All nests entrances similarly saw a

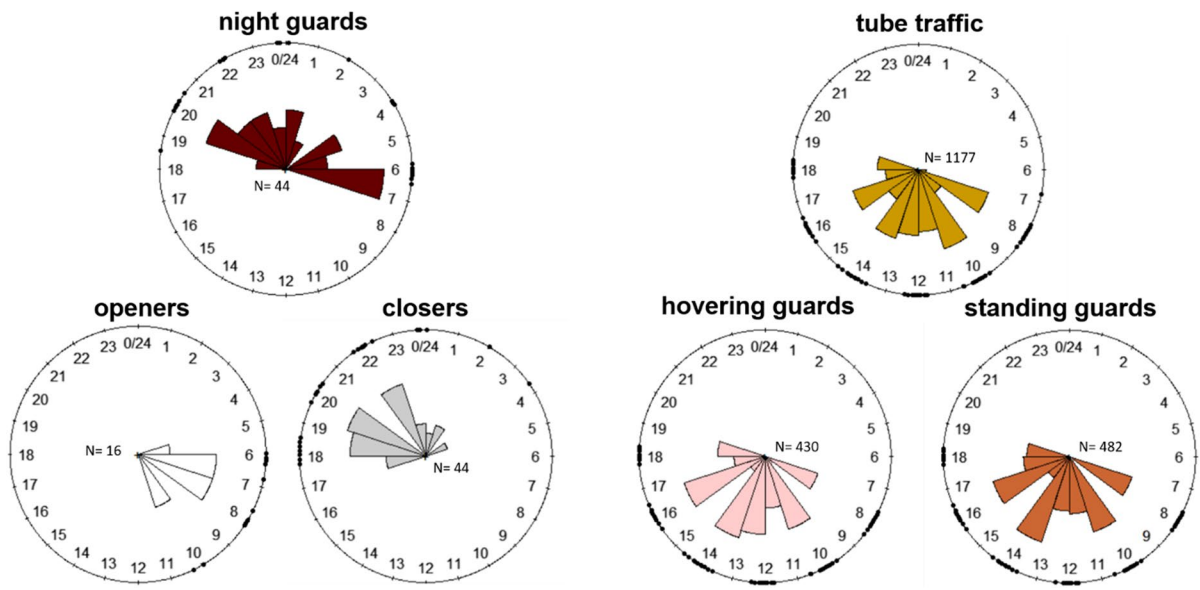


Fig. 1 Rose plots showing the frequency of bees performing different tasks throughout 24-h (openers, closers, night guards, hovering guards, standing guards and tube traffic). Foraging minors are assumed to be most of the tube traffic. Data is

pooled across six days, sampling every two hours. Axes are 24-h clock and bars represent the frequency distribution of a behavior

decrease in size between 18:00 and 20:00, with 40% remaining appreciably open the subsequent night. Although the area of nest entrance that was open or closed varied widely across colonies in day and night, these dawn and dusk windows of tube opening and closure appeared consistent across colonies (Figure S1).

The maximum number of guards we observed simultaneously standing at a nest entrance during evening hours was 4 and the minimum was 0. Although, the median number of guards observed across nights was 0 for closed nests (mean $0.406 \pm \text{S.E. } 0.910$) and 1 for open nests (mean $1.027 \pm \text{S.E. } 0.195$). This suggests that most nights had at least one guard standing at the entrance of open nests. Mean tube traffic as measured by foragers exiting and entering nests peaked at approximately 12:00 and decreased thereafter (Supplementary figure S2). We observed no foragers (tube traffic, Fig. 1) exiting or entering the nest after 20:00. Daytime standing guard behavior was unimodally distributed ($r_{\text{bar}}=0.9972$, Rayleigh test $p < 0.001$; Fig. 1), where they were most active midday at 13:65 (mean 13:19). Similarly, hovering guards were most active at 13:65 (mean 13:20), and unimodally

distributed ($r_{\text{bar}}=0.9987$, Rayleigh test $p < 0.001$). The median time we observed nest closing behavior was 20:13 (mean 20:50), and median opening behaviors occurred at 08:13 (mean 08:05), both were unimodally distributed ($r_{\text{bar}}=0.999$, Rayleigh test $p < 0.001$). Night guards median time of guarding occurred at 00:08 (mean 23:23) and was non-uniformly distributed (Hermans-Rasson test; $t=23.33$, $p=0.001$), and is possibly bimodal at approximately 20:00 and 06:00, dawn and dusk hours. Observing this bimodal pattern, we used a Hermans-Rasson of non-uniformity test, which is more sensitive to multimodality than Rayleigh tests (Landler et al. 2019).

Inter-Colony Differences in Nest Entrance Closure Across Nights

There were differences among colonies in closure patterns across the six observation nights (Fig. 2 bottom panel). Three colonies consistently closed on all six observation nights, two colonies consistently remained open on all nights, and the other 5 colonies intermittently closed and opened across nights. Colonies PK and ST (consistently closed), as well as SC and TR (consistently open) were

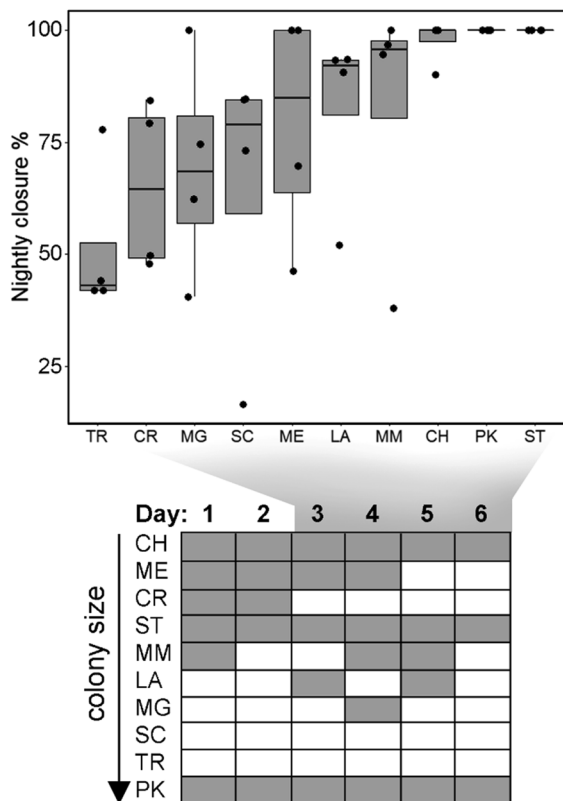


Fig. 2 (Above) Percent of nightly nest entrance closure across different colonies in this study observed across 4 sequential days. Nightly closure % significantly differed across colonies (repeated-measures ANOVA, $p=0.0112$). (Below) Closure of nest entrance tubes of 10 observation colonies across 6 days (the last 4 of which were also used for % closure measurements). White denotes nights on which the nest entrance tube bore at least one hole larger than the head of a *T. angustula* minor. Grey denotes nights on which nest entrances had no holes larger than a minor's head. Two-letter codes are colony IDs. Colonies are listed in order of increasing relative size (as estimated by maximum daytime traffic rate). The 6-day observations were used to select subject colonies for citral exposure (Fig. 5)

therefore chosen to use in subsequent manipulations testing whether a sensed obligate kleptoparasite kairomone (citral) can affect nest entrance closure. Colony ID was also a significant predictor of the degree to which nest entrance tubes closed each night as measured by percent closure from daytime nest entrance areas across 4 nights (Fig. 2 top panel; Repeated measures ANOVA: Colony ID $F_{9,29}=3.028$, $p=0.0112$; Time $F_{1,29}=6.73$, $p=0.0147$).

Identifying Morphological Caste of Night Guards

Sampled task-groups differed in size (Fig. 3; Mixed-model ANOVA: $X^2=126.29$, $df=4$, $p<0.001$). Night guards and tube closers were not different in mass (Tukey HSD; $z=2.167$, $p=0.1787$; Supplemental Table S1), but both were significantly larger than foragers Night guards $z=7.693$, $p<0.001$; Closers $z=-8.721$, $p<0.001$). They were also significantly greater in mass than soldiers performing diurnal defense tasks of hovering guarding (Night guards $z=4.254$, $p<0.001$; Closers $z=-3.080$, $p=0.0157$) or standing guarding (Night guards $z=-5.069$, $p<0.001$; Closers $z=-4.416$, $p<0.001$).

Night guards were few in number and relatively difficult to catch without destroying the nest tube. However, we achieved the capture and paint-marking of 8 night-guarding bees from two colonies that were then returned to their nests. We observed 2 paint-marked night guards performing the task of tube closing 24 h following marking, suggesting that at least some bees perform these behaviors for multiple nights. Interestingly, we observed 2 more marked night guards from two colonies, performing the daytime task of standing guarding 14 and 16 days following marking. We also observed a single paint-marked bee performing the task of hovering guarding 16 days following paint-marking. Given that the average length of time a single bee performs guarding behaviors is only about two weeks (Hammel et al. 2016), this together with the morphological data suggests that especially large soldiers may perform nighttime defense activities when they are fairly young, near the time when soldiers transition from internal nest activities to guarding behaviors. Anecdotally our data suggest that a small number of day guards also guard at night.

Tradeoff Between Tube-Closure and Night-Guards, but no Effect of Colony Size

Open nests had higher numbers of night guards than closed nests (Welch t-test: $t(66.081)=-2.4501$, $p=0.0084$; Fig. 4). However traffic rate (a proxy for colony size) was unrelated to colony average nightly closure (linear regression; $F_{1,8}=1.59$, $R^2=0.0612$, $p=0.2433$) and maximum night guard number ($F_{1,8}=0.17$, $R^2=-0.1017$, $p=0.6916$).

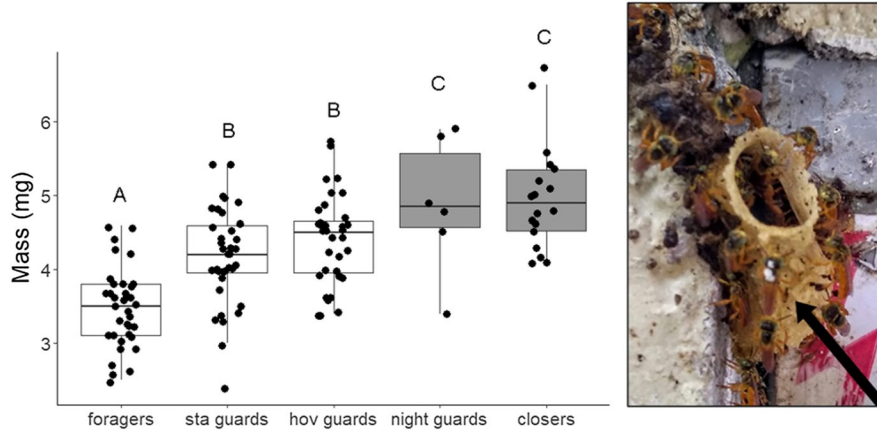


Fig. 3 (Left panel) A box-plot showing the mass of collected bees performing several tasks: foraging (known minors), standing guards and hovering guards (known majors), night guards, and nest entrance tube closers (the two focal nocturnal task groups of this study). Tasks performed during the day are shown in white. Tasks performed at night are shown in grey.

A mixed-model ANOVA with Tukey post-hoc comparisons showed differences in mass across task groups. Significant differences are denoted by different letters. All bees were returned to nests following weighing. Night guards were paint marked before being returned. (Right panel) A marked night guard performing standing guarding behavior during the daytime

Citral Application and Nest Closure

We tested the hypothesis that nest disturbance, simulated by the obligate kleptoparasite volatile citral, can affect nest closure. These preliminary data show that both nests which were reliably open throughout the night previously, partially closed following citral application (Fig. 5). Nest SC had an opening area of 0.182 cm² (20:00), that closed considerably by 02:00 to 0.003 cm². This 98% closure observed when citral was applied was far greater than the 1% closure observed for this colony over the same time interval on a night when no citral was applied. The open area of TR was 0.229 cm², which closed to 0.138 cm² by 02:00. This citral-associated nest entrance closure of 40% was also substantially greater than the 2% increase in nest entrance opening area observed over the same time interval on a night without citral application. Nests that were consistently closed, CH and PK, remained closed after citral application. We did not have enough reliably open nests to replicate sufficiently to conduct statistical analysis of these results, but these preliminary findings support the hypothesis that disturbance affects nest closure.

Discussion

Our data provide new insight into the nighttime defensive behaviors of *T. angustula* colonies. We describe the 24-h ethology of *T. angustula* activity at nest entrances, behaviors that have not yet been well described. We found considerable variation among and within colonies in tendency to close nest entrance tubes at night. It is thought that *T. angustula* close their nests at night to inhibit predation from nocturnal predators like ants (Roubik 2006), although this hypothesis has never been directly tested. Here we show preliminary evidence that kleptoparasitization pressure from other stingless bees may also be an important factor. Our data suggest that *T. angustula* colonies actively guard their nests at night with a smaller number of individuals, and that this task as well as tube closure may be performed by especially large soldiers. These data are some of the first to describe and quantify the nightly defense behaviors of *T. angustula* generating intriguing hypotheses for future study.

Our study described a diversity of defense behaviors being performed throughout the day and night at *T. angustula* nest entrances. Daytime guards, standing

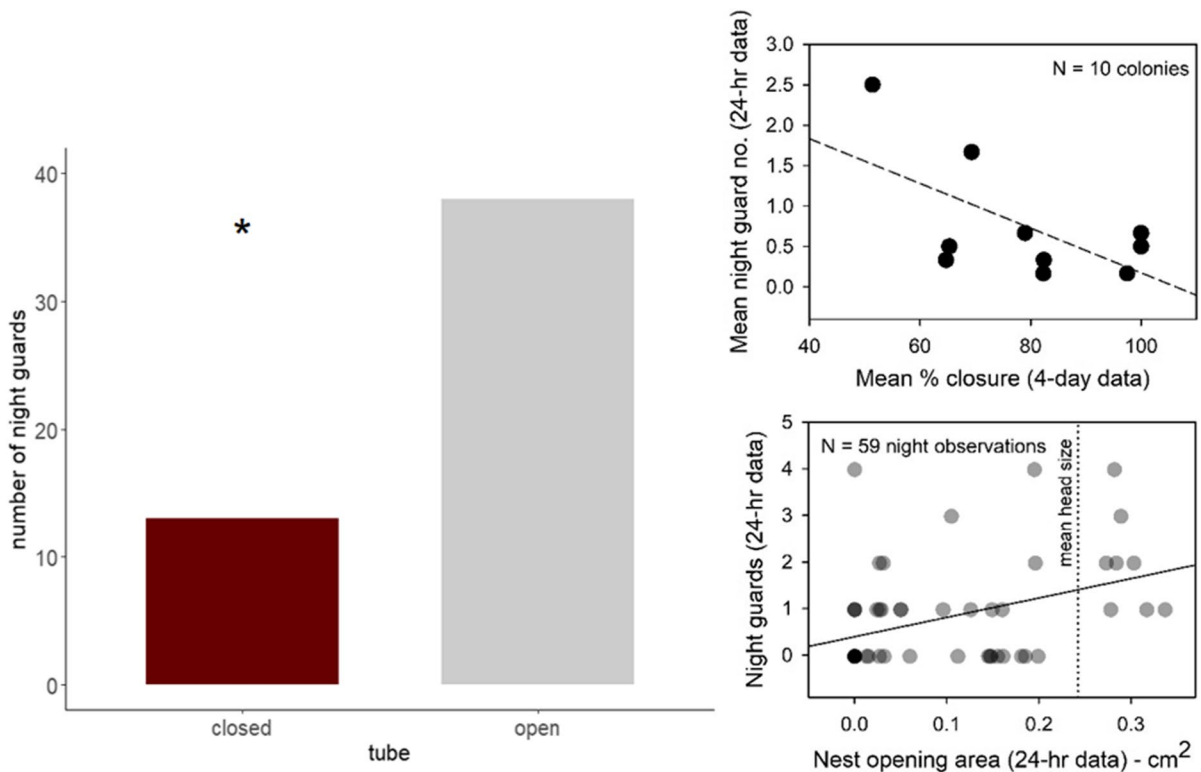


Fig. 4 (Left panel) Total number of night guards standing at the nest entrance pooled for 10 nests (replicated over 6 days). Bars show the number guards at closed and open nest tubes. Significance denoted by asterisk ($p=0.008$) using a Welch two sample t-test. (Right top panel) Colony mean night guard number as a function of mean percentage closure. The slope

was marginally non-significant ($F_{1,8}=4.81$, $R^2=0.2974$, $p=0.0596$). (Right bottom panel) Observation-wise correlation between nest opening area and number of night guards in a mixed-effect analysis that took colony ID into account as a random variable ($\chi^2=7.87$, $df=1$, $p=0.0050$)

and hovering, are most active between 10:00–18:00, which correlates with the timing of daily tube traffic, presumably mostly foragers. *Tetragonisca angustula* foraging occurs from the morning to late afternoon and is comparable to honey bees, although start time of foraging time shifts by region (Pernal and Currie 2001; Yucel and Duman 2005). We observed bees physically closing the nest (closers) after 16:00 and observed bees performing these behaviors up until approximately 04:00. Median opening time occurred at 08:13 and foraging seems to begin as soon as the entrance opens, peaking at approximately noon. After dusk, night guards begin their shift from 18:00–07:00. Perhaps night guards are most needed at dawn and dusk to fend off threats, or perhaps night guards and nest entrance tube manipulators are the same bees. Given how few bees within the colony were found to typically perform night guarding behaviors, future

studies with greater colony-level replication and longer-term observation periods are needed to test hypotheses related to specialization and allocation among these multiple nocturnal and diurnal defense task groups.

Across Hymenoptera, insect colonies display nest closure behaviors at night (Cane and Miyamoto 1979; Roubik 1992; Tofilski et al. 2008). Some ant, bee and wasp species physically close their nest entrances at night with soil or wax. In ant species *Forelius pusillus*, not only are nests shut at night but the last workers to shut the nest are also sacrificed, “locked out” of the colony (Tofilski et al. 2008). That *T. angustula* close the nest entrance tubes at night has been documented (Roubik 1992; Grüter et al. 2011), but very little is known about these behaviors. We found substantial variation in the likelihood of nightly nest closure across colonies, and for some colonies, variation

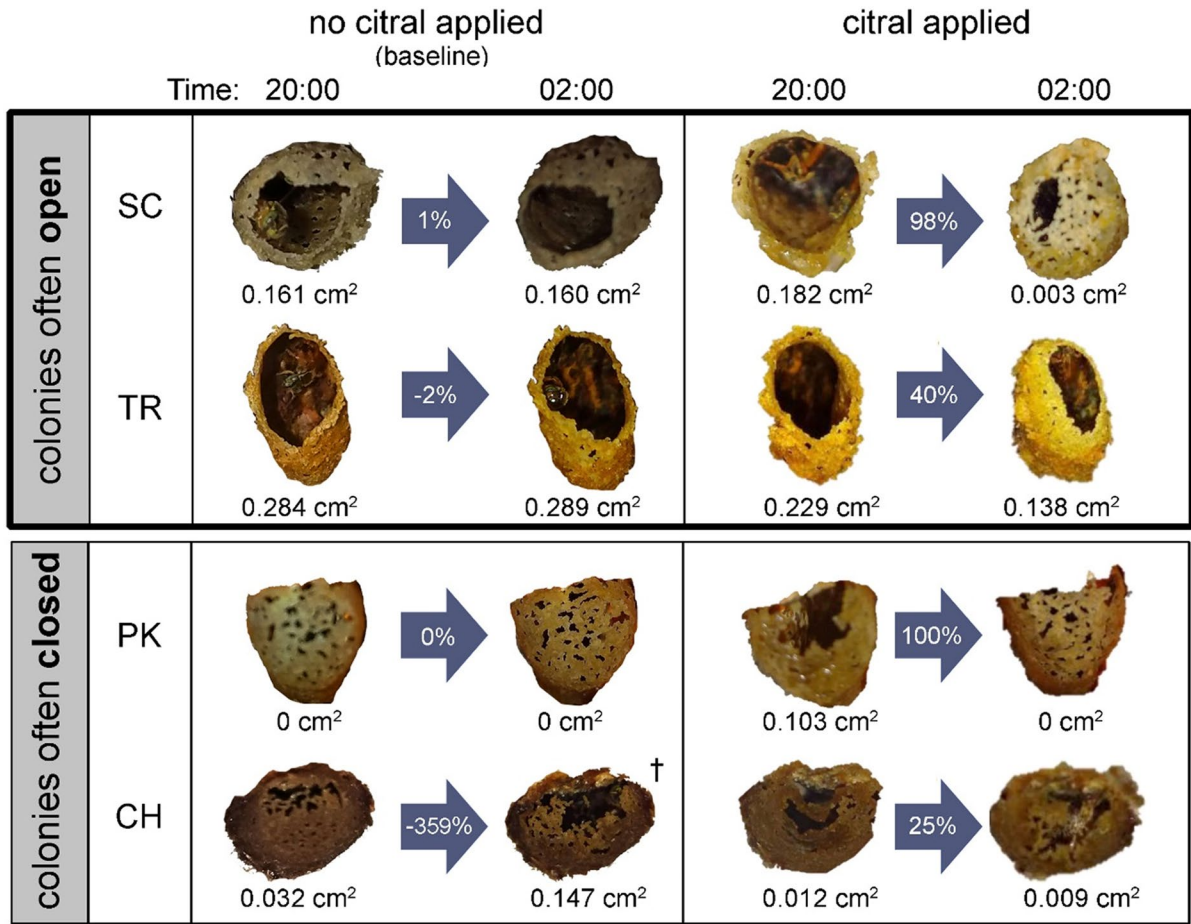


Fig. 5 Late night nest entrance closure of four colonies with and without artificial application of an obligate kleptoparasite volatile (citral) to filter paper next to the outside of the nest. We used colonies that were either open (SC and TR) or closed (PK CH) on all 6 immediately previous observation nights (Fig. 2). Baseline images at 20:00 and 02:00 (on the left) were taken from 24-h observations when no citral had been applied.

Citral was applied immediately following the 20:00 photo on the citral application night. Cross-sectional open area (cm²) for each nest entrance tube is beneath each photo. Percentage of closure between 20:00 and 02:00 is indicated within arrows. †The nest entrance of colony CH suffered nest entrance (possibly predation) damage independent of circadian tube opening

was also high across nights. To our knowledge, this is the first study attempting to quantify the variation in nest closure of this species. The wide variation in nest closure begs the question; what causes a *T. angustula* colony to close their nest at night? We found no evidence that colony size was predictive of nest entrance closure. We also tested the hypothesis that the presence of the karimone citral affects nest closure behavior, simulating nest robbing by obligate kleptoparasitic stingless bees (*Lestrimelitta* spp.). We observed that nests that were reliably closed, continued to close after exposure to citral. Interestingly, the nests that were reliably open on previous nights

closed their nests much more drastically after citral was applied. Results of this preliminary study support the hypothesis that sensed colony threats may affect nightly nest closure and may account for at least some of the observed variation within and among colonies in nighttime nest closure. This represents an exciting avenue for future investigation.

Whether nests are closed or left open, we found guards at the entrance of the nest tube many nights. Open nests had higher numbers of guards than closed nests. These data could suggest that open nest entrances are more vulnerable and need more behavioral defense. We verified that these bees were

morphologically distinct soldiers and not smaller minors by mass. Curiously, we found night guard and closer masses slightly greater than those recorded for daytime hovering and standing guarding soldiers, even though we observed marked night guards standing guarding and hovering guarding on following days. Together, this suggests that bees performing night guarding tasks are a subset of especially large-bodied soldiers which also perform diurnal defense tasks.

Our results support the hypothesis that there is a trade-off between night guard number and nest closure. Although this tradeoff appears to be independent of colony size, other colony characteristics or environmental conditions remain possible factors. A colony could conserve energy (resin foraging) by not closing the nest every night, only doing so when they experience a threat. Threats to nest entrances of *T. angustula* are also diverse, including ants, spiders, and other bees (Roubik 2006; Grüter et al. 2016; Ostwald et al. 2018). It is possible that high closure with few guards versus low closure with many guards are plastic strategies that can provide defense effective against different combinations of intruder types. We observed entrance tube opening size fluctuating and decreasing in size in some cases in the early morning hours. Colonies could be responding to disturbance in the middle of the night. For those that preemptively close, perhaps they are colonies experiencing daily disturbance. Another possibility is that nest closure behavior has a genetic component, where some colonies may be more likely to close than others. Numerous behaviors in honey bee colonies have been shown to be linked to heritable genetic variation such as aggression, hygienic behavior and cognition (Chandra et al. 2000; Lapidge et al. 2002; Sokolowski 2020).

Our study characterized the nightly behaviors at the entrance of *T. angustula* colonies. Our data suggest the degree of nest closure widely varies from colony to colony and, for some colonies, from day to day. We found no relationship between nest closure and colony size, but preliminary data showed that it is possible disturbance in the form of a sensed kairomone, citral, stimulates higher nighttime nest closure. We confirmed that night guarding bees are majors (soldiers) not minors, and that they likely perform nest closure and opening tasks. Furthermore, our study identified a possible trade-off between guard number and entrance tube closure. These data are a launching point for hypotheses in future studies examining nightly nest defense in stingless bee species.

Acknowledgements Research was conducted at the Smithsonian Tropical Research Institute in Gamboa, Panama. Permits for this research were issued by the Panamanian Ministry of the Environment (MIAMBIENTE). Funding was provided by the Arizona State University School of Life Sciences Innovative Postdoctoral Research Award to MMB and KMB.

Authors' Contributions MMB conceived the study. MMB and KMB designed the study, performed field work, collected data, and analyzed data. MMB and KMB both contributed to the final manuscript.

Funding Funding was provided by the Arizona State University School of Life Sciences Innovative Postdoctoral Research Award to MMB and KMB.

Data Availability All data used in analyses will be uploaded as supplementary upon manuscript acceptance.

Code Availability Not applicable

Declarations

Conflicts of Interest/Competing Interests Not applicable

References

- Balbuena MS, Farina WM (2020) Chemosensory reception in the stingless bee *Tetragonisca angustula*. *J Insect Physiol* 125:104076
- Baudier KM, Ostwald MM, Grüter C, Segers FHID, Roubik DW, Pavlic TP, Pratt SC, Fewell JH (2019) Changing of the guard: mixed specialization and flexibility in nest defense (*Tetragonisca angustula*). *Behav Ecol* 30:1041–1049
- Baudier KM, Bennett MM, Ostwald MM, Hart S, Pavlic TP, Fewell JH (2020) Age-based changes in kairomone response mediate task partitioning in stingless bee soldiers (*Tetragonisca angustula*). *Behav Ecol Sociobiol* 74:125
- Blum M, Crewe R, Kerr W, Keith L, Garrison A, Walker M (1970) Citral in stingless bees: Isolation and functions in trail-laying and robbing. *J Insect Physiol* 16:1637–1648
- Bowden RM, Garry MF, Breed MD (1994) Discrimination of con- and heterospecific bees by *Trigona* (*Tetragonisca*) *angustula* guards. *J Kans Entomol* 67:137–139
- Cane JH, Miyamoto MM (1979) Nest defense and foraging ethology of a neotropical sand wasp, *Bembix multipicta* (Hymenoptera: Sphecidae). *J Kans Entomol* 52:667–672
- Chandra SB, Hosler JS, Smith BH (2000) Heritable variation for latent inhibition and its correlation with reversal learning in honeybees (*Apis mellifera*). *J Comp Psychol* 114:86–97
- Dani FR, Jones GR, Corsi S, Beard R, Pradella D, Turillazzi S (2005) Nestmate recognition cues in the honey bee: differential importance of cuticular alkanes and alkenes. *Chem Senses* 30:477–489
- Gordon DM (1996) Soldier production under threat. *Nature* 379:583–584

- Grüter C, Kärcher M, Ratnieks F (2011) The natural history of nest defence in a stingless bee, *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), with two distinct types of entrance guards. *Neotrop Entomol* 40:55–61
- Grüter C, Menezes C, Imperatriz-Fonseca VL, Ratnieks FL (2012) A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee. *PNAS* 109:1182–1186
- Grüter C, Segers FH, Menezes C, Vollet-Neto A, Falcón T, von Zuben L, Bitondi MM, Nascimento FS, Almeida EA (2017) Repeated evolution of soldier sub-castes suggests parasitism drives social complexity in stingless bees. *Nat Comm* 8:4
- Grüter C, Von Zuben L, Segers F, Cunningham J (2016) Warfare in stingless bees. *Insectes Soc* 63:223–236
- Hammel B, Vollet-Neto A, Menezes C, Nascimento FS, Engels W, Grüter C (2016) Soldiers in a stingless bee: work rate and task repertoire suggest they are an elite force. *Am Nat* 187:120–129
- Jeanne RL (1975) The adaptiveness of social wasp nest architecture. *Q Rev Biol* 50:267–287
- Kärcher MH, Ratnieks FL (2009) Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition. *J Apic Res* 48:209–214
- Korczynski M, Hamieh A, Huh JH, Holm H, Rajagopalan SR, Feferman NH (2016) Hive oversight for network intrusion early warning using DIAMOND: a bee-inspired method for fully distributed cyber defense. *IEEE Commun Mag* 54:60–67
- Kurosu U, Aoki S, Fukatsu T (2003) Self-sacrificing gall repair by aphid nymphs. *Proc R Soc B P Roy Soc B-Biol Sci* 270:S12–S14
- Kwa HL, Tokić G, Bouffanais R, Yue DK (2020) Heterogeneous swarms for maritime dynamic target search and tracking. *Global Oceans 2020: Singapore-U.S. Gulf Coast* 1–8
- Landler L, Ruxton GD, Malkemper EP (2019) The Hermans-Rasson test as a powerful alternative to the Rayleigh test for circular statistics in biology. *BMC Ecol* 19:1–8
- Lapidge KL, Oldroyd BP, Spivak M (2002) Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. *Sci Nat* 89:565–568
- LaPolla J, Mueller U, Seid M, Cover S (2002) Predation by the army ant *Neivamyrmex rugulosus* on the fungus-growing ant *Trachymyrmex arizonensis*. *Insectes Soc* 49:251–256
- Miller EM (1969) Caste differentiation in the lower termites, vol 1. Academic Press, New York and London
- Ostwald MM, Ruzi SA, Baudier KM (2018) Ambush predation of stingless bees (*Tetragonisca angustula*) by the solitary-foraging ant *Ectatomma tuberculatum*. *J Insect Behav* 31:503–509
- Page RE Jr, Scheiner R, Erber J, Amdam GV (2006) The development and evolution of division of labor and foraging specialization in a social insect (*Apis mellifera* L.). *Curr Top Dev Biol* 74:253–286
- Pernal SF, Currie RW (2001) The influence of pollen quality on foraging behavior in honeybees (*Apis mellifera* L.). *Behav Ecol Sociobiol* 51:53–68
- Prestwich GD (1979) Chemical defense by termite soldiers. *J Chem Ecol* 5:459–480
- Roubik DW (1992) Ecology and natural history of tropical bees. Cambridge University Press, Cambridge
- Roubik DW (2006) Stingless bee nesting biology. *Apidologie* 37:124–143
- Schatz B, Wcislo WT (1999) Ambush predation by the ponerine ant *Ectatomma ruidum* Roger (Formicidae) on a sweat bee *Lasioglossum umbripenne* (Halictidae), in Panama. *J Insect Behav* 12:641–663
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671
- Seeley TD (1982) Adaptive significance of the age polyethism schedule in honeybee colonies. *Behav Ecol Sociobiol* 11:287–293
- Segers FH, Menezes C, Vollet-Neto A, Lambert D, Grüter C (2015) Soldier production in a stingless bee depends on rearing location and nurse behaviour. *Behav Ecol Sociobiol* 69:613–623
- Segers FH, von Zuben L, Grüter C (2016) Local differences in parasitism and competition shape defensive investment in a polymorphic eusocial bee. *Ecology* 97:417–426
- Seid MA, Scheffrahn RH, Niven JE (2008) The rapid mandible strike of a termite soldier. *Curr Biol* 18:R1049–R1050
- Shackleton K, Al Toufaily H, Balfour NJ, Nascimento FS, Alves DA, Ratnieks FL (2015) Appetite for self-destruction: suicidal biting as a nest defense strategy in *Trigona* stingless bees. *Behav Ecol Sociobiol* 69:273–281
- Shackleton K, Balfour NJ, Toufaily HA, Alves DA, Bento JM, Ratnieks FLW (2019) Unique nest entrance structure of *Paratamona helleri* stingless bees leads to remarkable ‘crash-landing’ behaviour. *Insectes Soc* 66:471–477
- Sokolowski MB (2020) Honey bee colony aggression and indirect genetic effects. *PNAS* 117:18148–18150
- Strickland LG, Baudier KM, Bowers KP, Pavlic TP, Pippin CP (2018) Bio-inspired role allocation of heterogeneous teams in a site defense task. *DARS. Springer Proceedings in Advanced Robotics*, vol 9. Springer, Cham
- Tofilski A, Couvillon MJ, Evison SE, Helanterä H, Robinson EJ, Ratnieks FL (2008) Preemptive defensive self-sacrifice by ant workers. *Am Nat* 172:E239–E243
- van Zweden JS, Grüter C, Jones SM, Ratnieks FL (2011) Hovering guards of the stingless bee *Tetragonisca angustula* increase colony defensive perimeter as shown by intra- and inter-specific comparisons. *Behav Ecol Sociobiol* 65:1277–1282
- Wilson EO (1968) The ergonomics of caste in the social insects. *Am Nat* 102:41–66
- Wittmann D, Radtke R, Zeil J, Lübke G, Francke W (1990) Robber bees (*Lestrimelitta limao*) and their host chemical and visual cues in nest defense by *Trigona* (*Tetragonisca*) *angustula* (Apidae: Meliponinae). *J Chem Ecol* 16:631–641
- Yucel B, Duman I (2005) Effects of foraging activity of honeybees (*Apis mellifera* L.) on onion (*Allium cepa*) seed production and quality. *Pak J Biol Sci* 8:123–126
- Zeil J, Wittmann D (1989) Visually controlled station-keeping by hovering guard bees of *Trigona* (*Tetragonisca*) *angustula* (Apidae, Meliponinae). *J Comp Physiol A* 165:711–718

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.