

ECOLOGY, BEHAVIOR AND BIONOMICS

The Natural History of Nest Defence in a Stingless Bee, *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), with Two Distinct Types of Entrance Guards

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Abstract

The stingless bee *Tetragonisca angustula* (Latreille) is the only social bee known that has two different types of nest entrance guards. As in other stingless bees and the honey bee one type stands on, in or near the nest entrance. The second type, so far only known in *T. angustula*, hovers near the nest entrance. In order to gain further understanding of this unique situation we studied guarding behaviour in both types of guards. Using marked bees, we found that individual worker bees guarded for a long time, up to 20 days, relative to their short, average c. 21 day, lifespan. Relatively few, 33%, individually marked guards were seen performing both types of guarding. The others only acted as standing guards. The bees that did perform both types did so over similar periods of their life. Hovering bouts were 57 min long, interrupted by breaks inside the hive of a few minutes (3.3 ± 1.5 min). Standing bouts were slightly longer (74 min) and also interrupted by short breaks (7.82 ± 6.45 min). Human breath, mimicking a vertebrate intruder, caused the guards to retreat into the nest rather than to attack the intruder. Some colonies protected themselves against intruders by closing the entrance during the night (32% and 56% of colonies during two nights). In summary, our results indicate that nest entrance guarding in *T. angustula* involves division of labour between the two types, in which most guarding individuals only act as standing guards.

Introduction

The nests of insect societies are defended by entrance guards (Butler & Free 1952, Wilson 1971, Roubik 1989, Hölldobler & Wilson 2009) who help protect the valuable resources inside, including adults, brood, food stores and nest material, and even the nest site itself. The guards admit nest-mate workers but exclude intruders. In social bees, food stores can be robbed by both conspecific and allospecific intruder bees (Michener 1974, Roubik 1989, Morse & Nowogrodzki 1990). For example, honey bee

colonies in Europe are often robbed by worker bees from other honey bee colonies (De Jong 1990, Downs & Ratnieks 2000). In addition, there is a small amount of robbing by bumble bees (FR pers. obs.). Stingless bees also rob both allospecifically and conspecifically, and in both tropical America and Africa there are obligate robber species, *Lestrimelitta limao* (Smith) and *Cleptotrigona* respectively, that rob both honey and brood food (Roubik 1989). Social bee nests are also subject to robbing by a wide range of other animals, from wasps to bears, and guards also defend against these intruders (Roubik 1989,

Morse & Nowogrodzki 1990).

The guards that defend a bee nest usually stand on, near or in the nest entrance (Butler & Free 1952, Wittmann 1985). The stingless bee *Tetragonsica angustula* (Latreille) is unique in that it has two distinct types of guards (Kärcher & Ratnieks 2009). As in the honey bee and other stingless bees there are standing guards, which in *T. angustula* are positioned on the inside and outside of the wax entrance tube. The other type, so far known only in this species, are hovering guards. Most hovering guards ("main group"; see Kärcher & Ratnieks 2009) hover within 20 cm of the entrance (see fig 1 in Wittmann 1985) facing the flight path in front of the entrance (Wittmann 1985, Wittmann *et al* 1990, Kelber & Zeil 1997). A few guards hover 20-50cm from the entrance, to the right or left ("side groups") (Kärcher & Ratnieks 2009).

Hovering guards attack allospecific intruders, particularly those with a volatile odour, such as workers of *L. limao* which smell strongly of citrus, or which are of a different colour than *T. angustula* workers (Wittmann *et al* 1990, Bowden *et al* 1994). The hovering guards force intruders to the ground by grasping legs and wings with their mandibles (Wittmann 1985, Bowden *et al* 1994).

However, hovering guards do not detect conspecific intruders (Bowden *et al* 1994). These are detected by the standing guards (Kärcher & Ratnieks 2009). Presumably, discrimination between nestmate and non-nestmate conspecifics is relatively difficult and requires the assessment of non-volatile chemicals present on the cuticle that need contact chemoreception.

Further information is needed in order to better understand this unique form of nest defence in bees. The aim of this study was, therefore, to collect data on the natural history of nest entrance guarding in *T. angustula* and to perform simple experiments in order to find out whether guards of one type switch to the other type of guarding (task switching) or if they specialize in performing one kind of guarding (division of labour; see Ratnieks & Anderson 1999 for definitions). We performed individual observations and estimated the duration of guarding bouts and the overall guarding time. In addition, we also investigated the response of guards to human breath, imitating the presence of a vertebrate predator, and closing of the entrance tube at night.

Material and Methods

Study site and species

The study was conducted in January and February 2006 and 2009 at Fazenda Aretuzina, a farm dedicated to the study of stingless bees and the conservation of Brazilian wildlife, near the town of São Simão, São Paulo

State, Brazil. The total observation time was about 60 days. At Aretuzina Farm, approximately 30 colonies of *T. angustula* are kept in wooden hives in the apiaries around the central farm buildings. In addition, there are approximately 10 colonies living in cavities in the walls of these buildings. *T. angustula* is a common species ranging from Veracruz, Mexico, to Misiones, Argentina (Michener 2007). In Brazil it is known as Jataí. Mature colonies in Costa Rica contained approximately 10,000 bees (van Veen & Sommeijer 2000).

Numbers of hovering and standing guards

We counted the numbers of hovering and standing guards during daytime by monitoring 15 colonies every 2h from approximately 8:00h to 20:00h on three study days in 2006. Counting was accurate because guards of both types move very little. For statistical analysis, we averaged the data collected over the three days for each colony. In order to relate the guard numbers to colony size, we used the foraging activity of colonies as an indirect measure of colony size. This is based on the assumption that larger colonies have more foragers. Foraging activity was measured by counting the bees entering the colony during 60 seconds.

Guarding duration

In order to estimate the duration of guarding duties we marked guards of both types and scan-sampled nest entrances four times per day (10:00, 12:00, 14:00, 16:00h) until no marked guards were seen for two days. To mark the bees, they were immobilized by putting them into a freezer (c. -4°C) for 6 min. We marked bees in two different ways. First, we captured guards of both types (from eight colonies) and group marked them using two different colours (acrylic paint) to indicate whether they were standing guards or hovering guards when collected. Additionally, we individually marked guards of both types using different combinations of dots of two colours (from seven colonies). Individual marking with acrylic colours was challenging because the bees are small (body length c. 5 mm; Wittmann 1985), but was necessary to investigate possible changes in guarding behaviour. A colour mark weighed c. 0.2% of the body weight (unpublished data). We discarded bees if the markings affected their ability of guards to fly, such as when the paint also marked the wings.

Individual observations

We observed individually marked guards (six hovering guards, 15 standing guards of seven different colonies) for 60 min and recorded the time spent guarding, the time inside the nest, and whether guards switch from one type of guarding to the other. The colour marks were easy to recognise on both types of guards and visible from several meters.

Guard reactions to human breath

Vertebrate breath can set off defensive reactions in both social (e.g. DeGrandi-Hoffman *et al* 1998) and non-social (Conner *et al* 1985) insects. However, *T. angustula* is a rather timid and small bee. For this reason we wanted to find out whether they attack, as do for example African honey bees (DeGrandi-Hoffman *et al* 1998), or retreat when exposed to breath. To quantify the reaction we counted the number of hovering and standing guards before and after exposure to human breath. We divided the hovering guards into one main group hovering close to the entrance (see introduction) and two side groups usually hovering separately on the left and right side of the hive. The application of breath was standardised by slowly breathing out through a plastic hose ($d \approx 5$ mm) from a distance of approximately 20 cm (distance measured from the end of the hose). For this experiment we used 15 colonies, treating them nine times each (three days x three trials per day). For statistical analyses, we averaged the data per colony.

Nest entrance closing

Colonies of *T. angustula* often close the tip of their entrance tube with a sheet of wax perforated with many small holes (< 0.5 mm) during the night, presumably to prevent nocturnal enemies from entering. The holes presumably aid in gas exchange. We monitored the entrances of 25 study colonies every 30 min in the evening (19:30-24:00h) and morning (6:30-9:30h) for two nights in 2009, with similar average temperatures (date 22.2°C date 21.9°C; 10 measurements every 30 min from 19:30-24:00h).

Statistics

We used both parametric and non-parametric tests to analyse our data in R 2.8 (R Development Core Team 2008). If transformations of the dependent variable were necessary to achieve a normal distribution and to stabilise the variance we used the box-cox method to find the best transformation (Crawley 2002). Descriptive statistics are given as mean \pm standard deviation. All tests are two-tailed.

Results

Numbers of hovering and standing guards

Guarding was most intense between 12:00h and about 18:00h (Fig 1). In this period there were about twice as many standing guards (14.3 ± 6.46 at 14:00h) as hovering guards (6.37 ± 4.64 at 14:00h). No bees were hovering at 08:00h and 20:00h, so standing seems to take place for a greater proportion of the day time (Fig

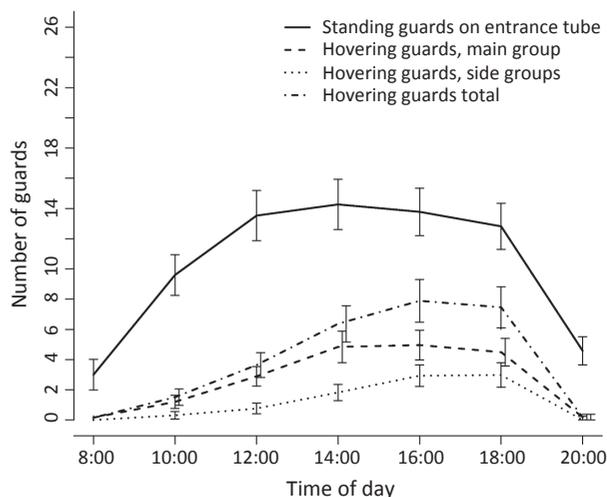


Fig 1 The number of standing and hovering guards (mean \pm SE), in both the main group and the side groups, at different times of the day ($n = 15$ colonies).

1). Hovering activity seems to be most intense during late afternoon. Fig 2 shows that there is a significant positive relationship between the number of standing guards and hovering guards per colony (Linear model (LM): cube root transformed data: $n = 25$; $F_{1,23} = 12.92$; $R^2 = 0.33$, $P = 0.002$).

We also found a significant positive relationship between the number of hovering guards and entrance traffic (number of bees entering in 60 s) (LM: cube root transformed data: $n = 25$; $F_{1,23} = 5.03$; $R^2 = 0.14$, $P = 0.035$). There was also a positive trend between number of standing guards and forager traffic, although this was not quite significant (LM: cube root transformed data: $n = 25$; $F_{1,23} = 3.4$; $R^2 = 0.09$, $P = 0.08$).

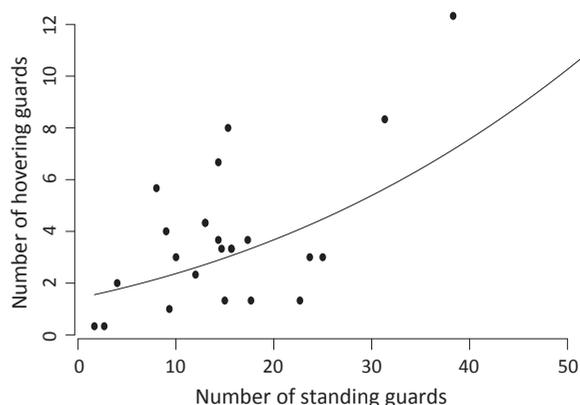


Fig 2 Relationship between the number of standing guards and the number of hovering guards. Each point represents the mean of three counts per colony ($n = 25$). There is a significant positive relationship between the two types of guards (LM: cube root transformed data: $n = 25$; $F_{1,23} = 12.92$; $R^2 = 0.33$, $P = 0.002$). Y-axis shows the untransformed data. The curve represents the best fit line.

Total guarding time

Data were collected from 46 marked bees. Fig 3 shows how many guards were observed during each day following the marking procedure and the type of guarding they did. The maximum guarding duration was 20 days (5.4 ± 5.48 ; Fig 3). The day following marking, more bees than expected were observed performing the role of standing guard. We had marked 49% hovering guards and 51% standing guards, then found that 91% were standing on the tube and 9% were hovering (Chi-square test: $\chi^2 = 28.5$, $df = 1$, $P < 0.001$). The same pattern can be seen during later days (Fig 3).

Individually marked bees

Of the 18 bees that were marked individually 12 were subsequently seen as standing guards, six performing both types of guarding, and zero exclusively as a hovering guard. Hence, 33% of all individually marked guards changed from one type of guarding to the other. Five of the six bees that changed duty during the sampling period were initially captured as hovering guards. We tested if bees initially captured as hovering guards switched from one task to the other more often than bees initially captured as standing guards. This was the case (Mann-Whitney U test: $W = 63$, $n = 8/10$, $P = 0.02$). Hence, some bees have a higher propensity to change tasks than others. The bees that changed task did so on average after 2.5 ± 2.04 days.

Individual observations

We observed 21 bees, each for a 60min period. During this time, hovering guards hovered for 56.7 ± 1.51 min ($n = 6$) and entered the hive 1.0 ± 0.0 times per bee. Hive stays were on average only 3.3 ± 1.5 min. Standing guards stood on the entrance tube for 54.2 ± 9.1 min, interrupted by 0.73 ± 1.09 mine hive stays per bee of 7.8 ± 6.45 min duration.

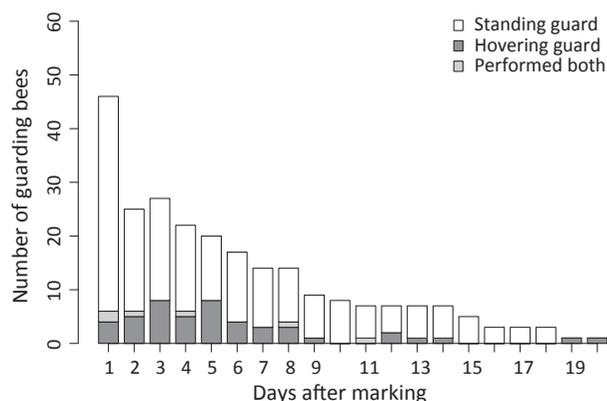


Fig 3 The number of individually marked bees performing guarding duties after marking. For each day the figure shows the number of bees still guarding.

In 21h of individual observations we never observed a bee switching from one type of guarding to the other. From the duration and rate of hive stays we estimated (by dividing the mean guarding duration per 60 min by the number of hive stays during the observation time) that single guarding bouts (time guarding between hive stays) are about 57 min for hovering guards and 74 min for standing guards.

Guard reactions to breath

After breath treatment, the number of hovering guards in the main and side groups decreased from 4.7 ± 4.02 and 2.2 ± 2.62 to zero, and the number of standing guards decreased from 12.2 ± 5.16 to 1.9 ± 2.54 . These changes are all highly significant (Wilcoxon-signed rank test: hovering bees main group: $V = 120$, $P < 0.001$; side group: $V = 91$, $P = 0.002$; standing guards: $V = 120$; $P < 0.001$; $n = 15$).

Geometry of entrance tubes

Each colony has a cylindrical or conical wax entrance tube made by the bees that was built into the hive entrance hole (The wooden hive boxes had a circular entrance hole 1.8 cm in diameter. Colonies had built a wax entrance tube from the inner surface of this hole). The tubes point slightly upward ($48.8^\circ \pm 9.27^\circ$ from horizontal; $n = 25$). As a result the lower length of the tube to the entrance hole was slightly longer than the upper length ($1.93\text{cm} \pm 0.87\text{cm}$ versus $0.81\text{ cm} \pm 0.73\text{ cm}$). The entrance was at the end and roughly circular (diameter $5.7\text{ mm} \pm 0.98\text{ mm}$, $n = 25$; Data from the colonies studied in 2009).

Opening and closing the entrance tube

On day 1 (2 February 2009), 14 of 25 colonies (56%) closed their nest entrance (sunrise: 06:46h; sunset: 19:54h local time). On day 2 (14 February 2009), eight of 25 (32%) colonies closed their entrance (sunrise: 06:54h; sunset: 19:47h local time; Fig 4). Ten of the 11 colonies that did not close on night 1 also did not close on night 2. However, this is not significantly different from randomness (Chi-square test: $\chi^2 = 4.73$, $df = 3$, $P = 0.19$).

Nest entrances were usually closed after sunset (11 of 14 on day 1, eight of eight on day 2). In order to be sure that colonies do not close their entrances after midnight we checked a sub-sample of the colonies at 02:00 on night 1 and again found two out of five colonies open. This suggests that there is no change in entrance status after midnight. The difference between night 1 and 2 in the proportion of closed entrances is not significant (McNemar test, $n = 25$, $P = 0.07$). Colonies with greater foraging activity were more likely to close their nest entrances (Mann-Whitney U

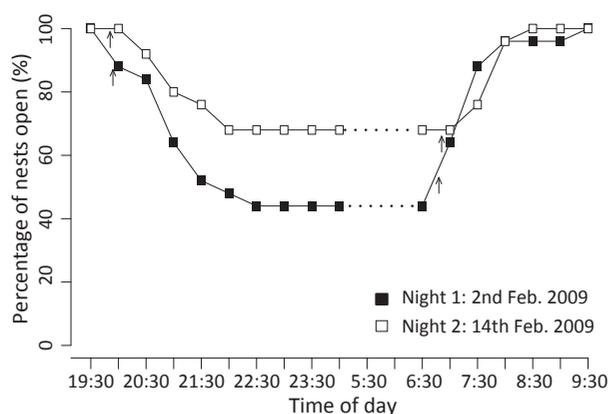


Fig 4 Proportions of nest entrance tubes that are open at different times of the day and night. The nest entrances were observed every 30 min at 19:30-24:00h and 06:30-09:30h during two different evenings and mornings. The arrows indicate the time of sunset and sunrise on both days.

test: night 1: $W = 116$, $P = 0.035$; night 2: $W = 101.5$, $P = 0.054$; $n = 25$).

Discussion

Our data give new information on guarding and nest defence in *T. angustula*. In particular, the results indicate that changing task from one type of guarding to the other is rare. The individually marked bees that did change from one type to the other (33% of all guards) did so on average every 2.7 days. About 66% only performed guarding as standing guards, while no guard was exclusively hovering. This suggests that hovering is performed by a sub-group of guards that change from standing to hovering. The low rate of changing tasks indicates that nest defence in *T. angustula* is an example of division of labour, rather than “task switching”, frequent switching between tasks as occurs, for example, during nest construction in *Polybia occidentalis* (Olivier) when workers switch between three different tasks, water foraging, pulp foraging and building (Jeanne 1986). Our data indicate that specialization in performing standing guard duties is greater than for hovering guard duties.

Although changing task was rare during observation periods, there is some indication that allospecific intruders induce standing guards to switch to hovering (Kärcher & Ratnieks 2009). Further study is needed to explore whether standing guards indeed switch to hovering for extended time periods after exposure to cues from allospecific intruders. We speculate that division of labour between hovering and standing guards increases the overall efficiency of nest defence. Indeed, the hovering guards effectively detect allospecific intruders (Wittmann 1985, Bowden *et al* 1994), making colonies almost immune against attacks of the cleptobiotic bee

Lestrimelitta limao (Smith) (P. Nogueira Neto, pers. commun). Hovering guards might also help the colony to detect allospecific intruders faster or at greater distance from the nest. Standing guards, on the other side, have remarkable abilities to discriminate nestmates from conspecific non-nestmates (Kärcher & Ratnieks 2009) making fewer errors than any other social bee previously studied (Breed & Page 1991, Downs & Ratnieks 1999, 2000, Couvillon & Ratnieks 2008).

Guarding duration

Tetragonisca angustula workers guarded for up to 20 days, with a mean guarding duration of 5.4 days (median = 3). This is longer than in European or African honey bees (*Apis mellifera*). In European honey bees guarding is performed by a small proportion of the workers, and most of those that do guard do so for less than one day (Moore *et al* 1987). The maximum guarding duration was around 6-7 days (Moore *et al* 1987, Hunt *et al* 2003). African honey bees may guard for up to 10 days (Hunt *et al* 2003). In both honey bees and *T. angustula*, most guards perform guard duties for only a relatively short period of time. The long guarding time in *T. angustula* might indicate a higher degree of specialization than in honey bees. Interestingly, Grosso & Bego (2002) found that *T. angustula* workers perform fewer tasks in their life-time than other stingless bee species, which suggests a relatively high degree of task specialisation compared to other species. The long average guarding duration is even more remarkable given that *T. angustula* workers live on average for only 20.9 days, which is the lowest mean life span found in stingless bees (Grosso & Bego 2002).

Observations on individuals show that guarding bouts (time between leaving the nest and entering the nest) are about 57 min for hovering guards and 74 min for standing guards. These bouts were interrupted by hive stays of 3.3 min for hovering guards and 7.8 min for standing guards. Presumably, hovering guards need to return to the nest to refuel but it is unclear why standing guards take nest breaks. The fact that standing guards take breaks suggests that they have more than a refuelling function. Taken together, the results of the focal and scan samplings suggest that bees often guard for many hours per day. Some bees can be observed standing or hovering at the same location from 10:00h to 16:00h for several consecutive days (C.G. pers. obs.).

We do not have information about the age of our guards but in other stingless bees as well as in honey bees guarding is performed towards the end of the age polyethism sequence, after completing in-hive tasks and before starting to forage (Winston 1987, Roubik 1989). As in other social insects, foraging in *T. angustula* seems to be the final task (Grosso & Bego 2002). On several occasions we observed returning bees (putative foragers)

with paint marks. With respect to the two different types of guarding, the results presented in Fig 3 do not suggest a particular type of temporal sequence with one type of guarding preceding the other. However, more detailed studies on age polyethism including guarding in *T. angustula* are needed.

Guard numbers

Colonies had more standing guards than hovering guards (Fig 1). This might simply be because standing on the entrance tube is energetically more affordable for colonies than hovering. On the other hand, if we assume that standing guards are more efficient against conspecific robbers (Kärcher & Ratnieks 2009), then higher numbers of standing guards might indicate a generally higher risk of conspecific robbing compared to allospecific robbing. There is evidence, however, that the number of hovering guards can be adjusted to the perceived risk of allospecific robbing. Standing guards perceiving odours of allospecific stingless bees often start hovering (Kärcher & Ratnieks 2009). Fig 1 indicates that hovering and standing guards have different activity peaks. While standing guards were most numerous from midday to late afternoon (12:00-18:00h; Fig 1), hovering guards were most active during the late afternoon. Is this caused by different activity times of potential conspecific and allospecific intruders?

We also found that the forager traffic of colonies positively correlates with the number of guards. This suggests that larger colonies also have more guards.

Reactions to breath

Eusocial bee nests are not only threatened by other bees but also by vertebrate predators (Roubik 1989, Kajoje & Roubik 2006) including humans, apes (e.g. *Pan troglodytes*), bears (*Tremarctos ornatus*), mustelids (e.g. *Eira barbara*), armadillos (*Priodontes* sp.) anteaters (*Tamandua* sp.), birds (e.g. *Indicator* sp.), toads and geckos (M. K., pers. obs.). Consequently, the breath from vertebrate predators elicits a defensive response in some bees (e.g. DeGrandi-Hoffman *et al* 1998). In *T. angustula*, breath did not cause aggressive defensive reactions. Instead, the number of guarding bees rapidly decreased as the standing guards retreated into the nest. During our experiments, guards of only one colony were occasionally aggressive against human observers that were standing close to the entrance for long periods of time. The bees of this particular colony tried to bite exposed skin. The nest cavities of *T. angustula* colonies probably provide a defensive barrier against these predators. Whether our results can be attributed to the small body size is not clear, since stingless bees of similar size can be aggressive against humans (e.g. *Plebeia pugnax*, F.L.W.R. pers. obs.)

Nest entrance closure

Many bee and ant species close their nest entrances at night to protect the nest against nocturnal enemies (Roubik 1989, Tofilski *et al* 2008). However, stingless bee colonies often do not close their nests completely (Roubik 1989). In our study population, approximately half the colonies closed their nest entrances in the evening after sunset. Furthermore, some colonies had partially closed entrances. Fewer colonies tended to close the nest-entrance after a day with a lot of rain. The temperatures were otherwise very similar. Nocturnal predators might be less active after heavy rainfall, thereby reducing the need to close the nest entrance. However, more data are needed to investigate the relationship between weather and nest entrance closure.

Tetragonisca angustula has an intriguing nest defence organisation with two distinct types of nest entrance guards (Kärcher & Ratnieks 2009). Although our results make a contribution to understanding guarding behaviour and nest defence in *T. angustula*, perhaps their main value is in directing future research in relation to some of the major questions. For example, why does there appear to be division of labour rather than task switching? What is the advantage in having hovering guards? Why do some colonies not close their entrances, and how is entrance closing organized?

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