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Studying the Biological Characteristics of the Stingless Bee *Tetrigonilla Collina* Smith 1857 (Apidae: Melipona) in Hanoi, Vietnam

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Abstract

Stingless bees are distributed in tropical and subtropical regions. There are over 16,000 species of stingless bees around the world, among which, 42 species are from Asia (Kerr & Maule, 1964). Sixteen species of stingless bees have been identified in Vietnam (Sakagami, 1975; Sakagami, 1978; Engel, 2000; Chinh *et al.*, 2005; Rasmussen, 2008). In this study, the biological characteristics of *Tetrigonilla collina*, as a representative for Southeast Asian stingless bees, were observed and recorded. The worker cell dimensions were 5.30 ± 0.34mm in length by 4.28 ± 0.37mm in width with a volume of 45.02 ± 5.74µL (food volume: 19.05 ± 3.5µL; ratio: 42.27%). The new cells were mostly built in the morning (from 2:00 to 10:00 am) and the queen laid eggs (2.52 ± 0.81 sec per egg) and capped the cells in the afternoon (from 12:00 to 17:00 pm). The average worker-cell building period was 12.03 ± 3.78 hours. Mainly, six of first ten bees that inserted made food discharges in a cell. The food discharge occurred in a very short time, which was 1.58 ± 0.6 minutes. The new cell numbers of the colony were reduced at the end of the experimental period.

Keywords

Stingless bees, *Tetrigonilla collina*, biology, Vietnam

Introduction

There are about 20,000 different bee species worldwide, varying in size, form, and lifestyle (Velthuis, 1997). Among them, stingless bees and honeybees together are on the highest level of bee social evolution. It is known that stingless bees are one of the oldest among the superfamily of the Apoidea. According to Velthuis (1997), stingless bees were once distributed on all continents around the world, which was the result of continental drift. Currently, stingless bees can be found in tropical and subtropical regions. South America

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is considered the center of origin and dispersion of stingless bees, which has 183 species, while Africa has 32 species, Asia has 42, and Australia, New Guinea, and the Solomon Islands have 20 (Kerr & Maule, 1964). In Vietnam, six species were recorded in the South by Sakagami (1975; 1978), Engel (2000) and Chinh et al. (2005) recorded two species in the North, and Rasmussen (2008) recognized 8 species. In total, about 16 species of stingless bees have been found in Vietnam, which include diverse topographies and varied climates.

Stingless bees play an important role in pollination, working well within crop-ecosystem interactions (Kwapong *et al*., 2010). Slaa *et al*. (2006) has emphasized that stingless bees are effective pollinators in a large number of crops. For instance, *Trigona (Lepidotrigona) terminata* Smith, 1878 (Hymenoptera: Apidae: Melipolini) is the most productive pollinator of coffee trees (*Coffea arabica* and *Coffea canephora)*. Additionally, stingless bee products are considered better than honeybee products. For example, the antioxidant properties in the honey of stingless bees are higher than those of honeybees (Kek *et al.*, 2017). In addition, a stingless bee's propolis is considered a potential ingredient for medicines. By using extracted propolis from four stingless bee species, practicing on five cell lines, Kustiawan *et al.* (2014) pointed out that propolis from *Trigona incisa* and *Trigona fuscobalteata* contained protective substances against human cancer. In other research on the propolis of *Lisotrigona cacciae*, the collected sample was considered a valuable product as well as a promising source of biologically active compounds (Georgieva *et al*., 2019).

Stingless bees make nests in cavities, and the nest entrance varies in shape, length, and color (Kelly *et al*., 2014; Syafrizal & Yusuf, 2014). The typical structure of nests consists of entrance tunnels, brood cells, food storages (honey and pollen cells), cerumen, and batumen layers (Sakagami *et al*., 1983; Starr & Sakagami, 1987; Chinh & Sommeijer, 2005; Michener, 2007; Boongird, 2011). Although some biological characteristics of stingless bees have been researched in different parts of the world, studies of the same bees in Vietnam are much more limited (Chinh *et al*., 2005; Chinh & Sommeijer, 2005). *Tetrigonilla collina* was reported in South of Vietnam by Sakagami & Yamane (1978), but it was first recognized in the North of Vietnam. The nest of this species is typically underground inside a termite colony. Other biological information about *T. collina* has not been studied in Vietnam yet. Researching the biological characteristics of stingless bees is very important for conserving them as pollinators and keeping them for the purpose of poverty alleviation.

Materials and Methods

Collection and handling of the Tetrigonilla collina for study

Three colonies of *Tetrigonilla collina* were collected from Noong Het commune, Dien Bien District, Dien Bien province (GPS: 21º18'02''; 103º01'40''; 510m) and moved into an insectary on Vietnam National University of Agriculture's campus in 2019, which is about 600km away. This species was first recognized in the North of Vietnam. The nest of this species was found 1.5m underground inside a termite colony.

Brood cells and eggs measurements

Cells were dissected daily by scissors right after the queen laid eggs. We then cut on the pillars which connect the cells and keep the brood cells firm. All the cells used in the study were undamaged and undistorted. After dissecting, the cell size and egg size were measured using a digital video eyepiece (DVE). Thirty-one random cells with their eggs were selected in the study.

Intact cells were kept vertically on a slide. Egg removal was done before making a 1mm-diameter hole on each cell. This work was performed carefully to avoid cell wall distortion. A 0.5-10µL micropipette was used to fill the cells with water to measure cell volume. The brood-food volume was determined by subtracting the free space volume from the cell volume.

Oviposition process

All of the activities during the oviposition process were recorded using a Logitech HD C615 Webcam. The duration of cell building, egg-laying, and cell sealing were then marked/noted and transcribed for further analysis. The recording began at 7:00 pm until the queen finished laying eggs the day after. Team Viewer software was installed on a mobile phone to remotely control the laptop.

Cell building and egg laying

We collected, built, and sealed in one batch of cells weekly. The total number of cells built was counted before oviposition while the number of eggs was observed after oviposition. The temperature and relative humidity were recorded daily at the experiment station.

Data analysis

All data in this research was analyzed by using Minitab Statistical Software ver. 16. Mean ± standard deviation (SD) was used for descriptive statistics. For comparison of means, t-Test was used with P-value less than 0.05.

Results and Discussion

Worker's brood cells

T. collina was found underground and their nest formed a cluster style of brood cells. This type of brood worker cell accounts for the high construction for natural building behaviors. There were two sections inside the nest: the brood zone and the bigger storage zones. However, a small number of brood cells could still be found in the built-in storage zone, close to the pots. Both sections were made from black soft cerumen. Nevertheless, the brood cell color changed from black to transparent yellow cocoons by removing the wax cell walls and making pupae visible, while the color and status of the storage cells were maintained. In terms of brood sessions, pillars play an important role in cell building. Workers constructed the pillars not only to link cells to other cells, but also to hold clusters of cells to the wall. Moreover, the pillars work as bridges to set the cell collars, thereafter, creating the cell foundation (Jongjitvimol & Wattanachaiyingcharoen, 2007).

Cell dimensions

Brood cells were built clinging onto the box wall by pillars. Workers constructed the globular brood cells. **Table 1** shows the measurements of the 31 cells, in which the average cell length was 5.3mm and average cell width was 4.28mm. The disparity of the length and width was significant ($P< 0.001$), therefore the size of the cells was oval style. The brood structure was a cluster by observations. This means that this stingless bee species does not construct horizontal combs.

Compared to the worker pot size of another stingless bee species, which was previously published by Roopa *et al.* (2015), the brood-pot dimensions made by *Trigona (Tetragonula) iridipennis* were noticeable different. The brood cells' height and width of the studied colonies ranged from 2.1 to 2.7mm and 1.7 to 2.0mm with averages of 2.5 and 2.0mm, respectively. These measurement values were much smaller than those of *T. collina.* The *Trigona collina* brood worker cells were bigger than those of *L. capenteri* (3.1 x 2.1mm) and *T. laeviceps* (4.5 x 5mm) (Chinh *et al.,* 2005). The total body length of *T. collina* was around 5.7mm, larger than that of *L. capenteri* and *T. laeviceps,* with lengths of 3.2mm and 4.9mm, respectively. The brood workers' cell dimensions were highly correlated to body size.

Workers regularly checked the cells once operculated. This behavior was mentioned in the

Table 1. Worker cell dimensions, cell capacity, brood-food volume, and the ratio of brood-food of *T. collina* (n = 31)

Length (mm)	Width (mm)	Capacity (μL)	Brood-food volume (μL)	The ratio of brood-food (%)
5.30 ± 0.34	4.28 ± 0.37	45.02 ± 5.74	19.05 ± 3.5	42.27 ± 5.44

Note: Mean ± SE.

report of Chinh *et al.* (2005), who described the polishing of cells by workers. This phenomenon was defined by wax removal. Simultaneously, workers could open the cells and take out the eggs, or even larvae or pupae, if they were spoilt for some reason.

Cell volume

The brood food was a yellow liquid, which became condensed after some time and changed color to orange. Different from *Apis* spp., the brood cells were not reused in the next egg-laying batch. In this study, we measured a total of 31 cells. Cell volumes ranged from 38.5µL to 56.0µL. Furthermore, we found that the workers discharged about 12µL to 27µL of brood food into a single cell, which occupied from 30 to 52% of the total cell capacity. Similar to cell size, the volume of brood food varied among different species. For instance, according to Rosa *et al.* (2015), the brood food volumes of *M. obscurior* and *S. bipunctata* were greater than that of *T. collina*, with values of 49.8µL and 37.3µL, respectively. In contrast, the amounts of larval food of *P. droryana* and *T. fiebrigi* were 9.4µL and 10.1µL, respectively, smaller than that of *T. collina.*

Oviposition behaviors

Fixation phase

In this study, the fixation phase of the cell building process was recorded from the initiation of pillars until cell completion. A total of 34 cells were recorded (**Table 2**). The mean duration for a single cell record was 12 hours. During this phase, workers not only built the cell height but also smoothed and polished the inside and outside of the cell. In addition, workers would remove the entire cell whenever it was distorted or damaged.

The *T. collina* queen laid eggs in the afternoon and then the cell construction began at night. Almost all the cell construction in a batch began at different times during this phase, therefore, rapid construction occurred on some cells that had begun late. The minimum time of cell-building was 6 hours, and the maximum was 17 hours (**Table 2**). The beginning time of cell-building was variable. However, even early or late, the cell building process was completed for the queen to lay eggs in the afternoon. All our results supported the research of Sommerijer *et al.* (1984).

Following cell building, the process was divided into six stages based on the mean high as illustrated in **Figure 1**. In the fixation progress, there were always two or more workers building from the beginning to the completion of a single cell. Some behavior patterns of workers were also observed.

First, a worker's body rotated around a cell with her mandibles building the collar of the cell steadily. This pattern was performed from the start of cell construction until ¾ of each cell had been built. From the ¾ built stage onwards, there were more building styles of workers observed. In one style, a worker climbed into a cell and inserted a metasomal tip, began rotating, and the cell was built higher by her mandibles. Another pattern of cell construction was polishing the outside of the cell. All the bodies of the workers clung onto the outside walls of the cells and polished. Workers also polished the cell orifice when it was high enough. Both building and polishing could be performed in a single cell simultaneously. During this phase, the queen was recorded inspecting the cells infrequently.

Table 2. The average time of cell-building, time of food discharging, and number of workers per cell

Parameters	Duration of		# Discharging workers per cell (individual)
	Cell-building (hours)	Food discharging (minutes)	
n	34	46	46
Min	6.0	0.73	4
Max	17.0	3.03	91
Mean ± SD	12.03 ± 3.78	1.58 ± 0.60	38.08 ± 25.82

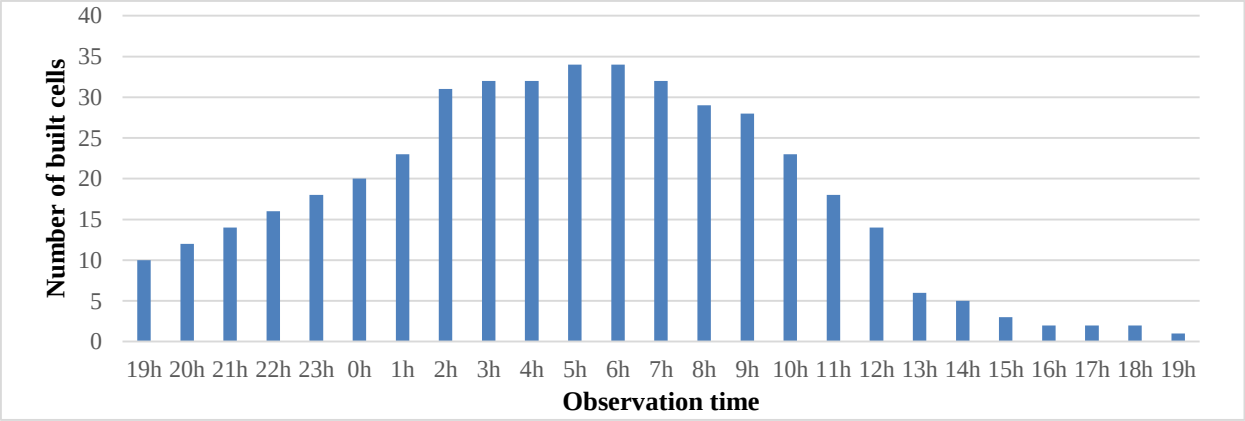

Figure 1. Number of cells built during 24 hours of observation

The density of cell building at selected times is shown in **Figure 1**. The highest building frequency was from about 5:00 am to 6:00 am, with almost 35 cells built. The number of cells built increased from 7:00 pm to 5:00 am and then decreased from 6:00 am to 7:00 pm. This phenomenon could be explained in that the period from 7:00 pm to 5:00 am was the time the bees did not fly out of the nest, so they spent this time working inside-jobs, such as cell building. Based on this record, we could conclude that the number of cells built was not high in the daytime. The highest period of cell-building lasted from 2:00 am to 7:00 am, with up to 30 cells each hour.

Each cell was constructed by several workers. The cells were not identical, thus we randomly chose cells in various stages at a selected time. At the starting stage, bees were forming the cell collars. The number of cells was highest at the ¼ height status, with 6 and 7 cells at 4:00 am on August 19th and September 2nd, 2019, respectively. Cell building could not be continuous. Workers almost finished their construction at the nearest batch for oviposition. However, there were a number of cells that were not completed, even after several oviposition periods. This situation was also mentioned in the report of Sakagami & Yamane (1987), who researched *T. (Lepidotrigona) ventralis*, with the same type of cell construction. There was also a case in which the operculated cells at the margin were had their contents ingested to construct new cells in Sakagami & Yamane (1987).

The line graph of **Figure 2** shows the quantity of cells that were initiated and completed over a 24 hour period, with the blue line and orange line, respectively. From an overall perspective, one of the most outstanding features was that the highest number of cells that begin to be built was 10 cells at 7:00 pm, followed by 8 cells at 2:00 am. This means that at two time points, workers were promoted in cell construction. Moreover, no cell began being built from 6:00 am to 8:00 pm. In contrast, there was an upward trend in the number of cells that were finished. Cells were completed just from 7:00 am to 8:00 pm. All the cells were not completed at the same moment. The peak number of completed cell buildings was at 1:00 pm.

Provision phase

This stage began when the first worker inserted into a cell to discharge food until the egg-laying moment of a queen. Food discharge occurred in a very short time (**Table 3**), with the average time being 1.58 minutes. Of note, the fastest time workers spent to fill one cell was just 44 seconds (0.73min).

Normally, workers congregated around a brood cell right at the start of the food-discharging phase, then moved quickly from cell to cell. In contrast to the cell-building phase, worker congestion was a signal of brood-food discharging. The order of food-discharging in cells was nonruling. During the provisioning phase, workers attended the cells not only to discharge food but also to check a cell's status. For behavior observation, there were no cells that began discharging at the same time. There was always the queen's first oviposition at the first

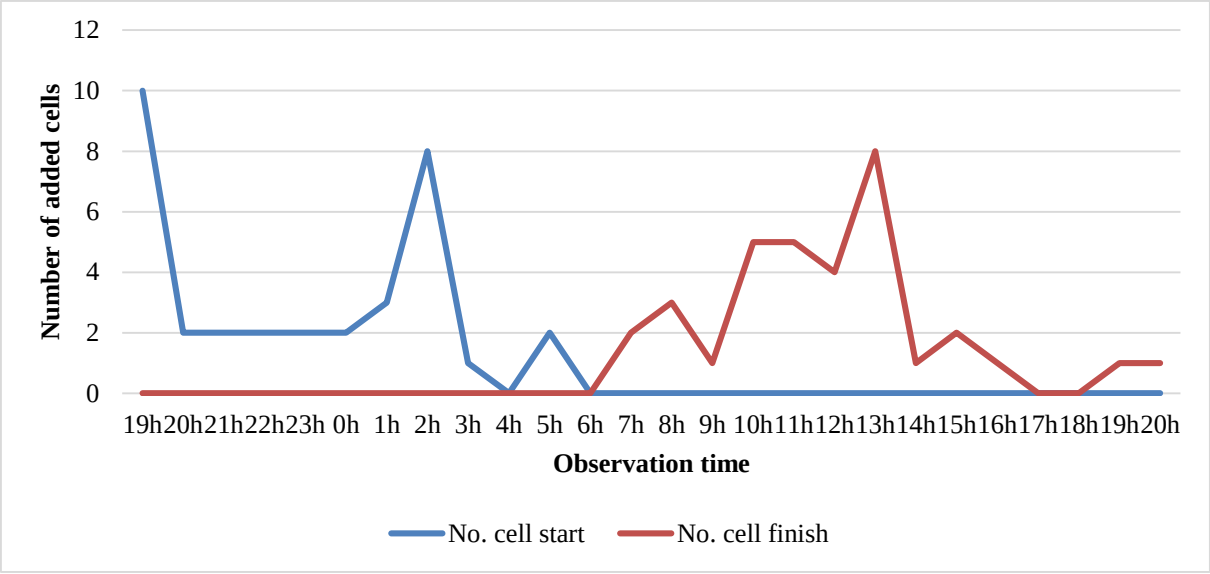

Figure 2. The number of cells added throughout the period of 24 hours

Table 3. Insert duration of the first 10 workers who discharged food into a cell (seconds)

	Min	Max	Mean ± SD
1st inserted worker	7.11	24.35	15.72 ± 4.25
2nd inserted worker	5.62	18.74	11.45 ± 3.75
3rd inserted worker	0.58	16.82	7.23 ± 3.10
4th inserted worker	0.41	10.41	5.99 ± 2.09
5th inserted worker	0	8.94	4.49 ± 2.56
6th inserted worker	0	9.94	3.04 ± 2.68
7th inserted worker	0	8.79	1.50 ± 1.64
8th inserted worker	0	9.05	1.29 ± 1.35
9th inserted worker	0	2.91	1.11 ± 0.63
10th inserted worker	0	1.95	0.92 ± 0.46

cell, which was followed by discharging in each cell in rapid succession but did not always follow after the prior-cell oviposition. This type of provision phase was similar to the semi-synchronous (D_m) phase described in the report of Sakagami & Yamane (1987). As for queen behaviors in this stage, there was a significant difference with the observations of Sakagami (1982) on the *Melinonula* queen. The *Melinonula* queen was absent during the gradual increase of workers in the food provisioning stage while the presence of the *T. collina* queen was the initiation of food discharging.

The food provisioning occurred in a very short turn in each cell, ranging from 0.7 minutes to 3.03 minutes. Many workers discharged jelly into a cell (**Table 2**), but the first ten workers who inserted into a cell during the food-discharging phase were investigated and measured (**Table 3**). The first worker who inserted into the cell was the longest discharging bee. There were at least four workers who inserted to discharge brood food. The highest number of workers who inserted to discharge food was eight. Those who had an insert duration of less than three seconds were considered to be checking on the cells after enough brood-food had been added.

The checking insertion made by workers was performed until the queen laid eggs. Therefore, the longer the time it took before the brood-food

was filled, the larger the number of workers who inserted into the cell. Once the cell was provided with an essential amount of food, workers continued to perform head insertions. This behavior continued until the queen approached that discharged cell. When there was no cell ready for laying eggs, the queen either passed the cells or waited. In some cases, the queen did not lay eggs when there was not enough brood food. The workers then immediately came back to fill the cell until the queen accepted.

Oviposition phase

The oviposition phase was counted from the first egg-laying time of the queen to the last ones in one batch. Once all the food-discharged cells were provided with an egg, the queen continued cruising the rest of the cells for a short time. She never missed a provisioned cell. However, there were some completed building cells that the queen did not lay eggs in because workers did not discharge food in these cells. The duration of oviposition was 2.52 seconds per cell (**Table 4**). This duration was higher than that of *T. clavipes*, with only 1.9 seconds (Sakagami & Zucchi, 1967). Furthermore, the egg-laying duration in each cell was not significantly different from *L. ventralis* (Sakagami & Yamane, 1987). The mean period of queen egg-laying in a single-cell is provided in **Table 4**.

The observed oviposition process involved the queen putting her body over the provisioned cell, inserting her metasomal tip inside the cell, and making a single pulse. In general, the oviposition process always occurred in a batch with a stable number of cells, and intervals between batches were large enough and stable at once per day, which was similar to the exclusively batch type (B_e) (Sakagami, 1974). However, in the first few days of observation, the oviposition process formed the batch loosely (B_f). The interval between batches was variable. In this case, oviposition occurred twice per day with the total number of cells per batch being 1 to 3 cells.

Worker eggs were taken out immediately after the queen oviposited. A single egg was laid per cell, standing up on the surface of the worker food liquid. These eggs were smooth and creamy white with a transparent membrane that kept the egg firm and stable in a columniform shape. After a few days of absorbing the food liquid, this membrane became thinner and thinner, which was then broken easily for hatching.

In comparison with the egg dimensions of *T. iridipennis* Smith (Roopa *et al.*, 2015), a *T. collina* egg was smaller, with a length of only 0.77 mm and a width of 0.3 mm (Table 5). The deviation of egg length and egg width was quite stable.

The appearance of laying workers in orphan colonies is a normal event in social insects. Remarkably, worker oviposition was not observed at all, even when the queen disappeared. After the "escape" of the queen, all the workers maintained their cell-building status and there were no workers laying eggs.

Operculated phase

The term "operculation" or "operculated phase" were used to indicate the worker's process in which they sealed the cells after the queen laid eggs. Routinely, operculation followed immediately after the queen oviposited, but it was sometimes delayed for a few seconds. In general, there was one worker who operculated discontinuously per a single cell.

Table 4: Egg-laying duration, egg dimensions, operculation time, and the number of workers who operculated

Parameters	Egg-laying duration per egg (seconds)	Egg dimensions (mm)		Operculation	
		Length	Width	Operculation duration in a single cell (min)	Number of workers who operculated
n	39	31	31	46	46
Min	1.55	0.72	0.27	16	1
Max	4.77	0.82	0.34	32.02	4
Mean ± SD	2.52 ± 0.81	0.77 ± 0.03	0.30 ± 0.02	23.89 ± 4.40	1.74 ± 0.99

The operculating worker built from the cell orifice upward (**Table 4**).

Only when the cell was closed, except for a small enteral hole, might another worker join in completing the operculation. As in most behaviorally known stingless bees, cell operculation consists of rotation and side work (Sakagami & Zucchi, 1967). Both patterns were observed in *T. collina*. At the beginning of the operculation process, a worker climbed on the cell, and started closing it from the orifice cell by inserting her metasomal tip into the cell and rotating. Then the operculator withdrew her metasoma from the cell and remained on the outside of the cell alone or with other workers. The closure time for each cell was counted from when the first worker began closing the orifice cell until the cell was totally sealed. There was a minimum of one worker to a maximum of four workers involved in the cell sealing process. Moreover, the operculation interval of the *T. collina* queen was longer than in observations by Sakagami (1982) on *Scaura*, *Tetragona*, and *Trigonisca*. These durations were 1 minute in *Scaura*, and 11 minutes to 12 minutes in *Tetragona* and *Trigonisca*.

Dynamics of the oviposition process

The number of cells built over nine days is shown in **Figure 3**. The interval between each observation was 7 days. The temperature and relative humidity were recorded.

Due to the lack of involucrum, high temperatures had the strongest impact on the *T. collina* colony. In nature, the *T. collina* colony was found about 2m underground inside a termite nest. When it was too hot, the emergency rate of the eggs was very low, compared to cool days. After transferring the bees to an observation box, workers swiftly coated all the inside areas of the wooden box wall with black brown cerumen. However, it was too thin and just used for sealing the slits. There was some proof that workers speedily nibbled this matter from the wall and then set it on the opened slots after the glass lid was recovered. Therefore, this thin layer could not keep the optimal temperature inside the nest for this species. The number of

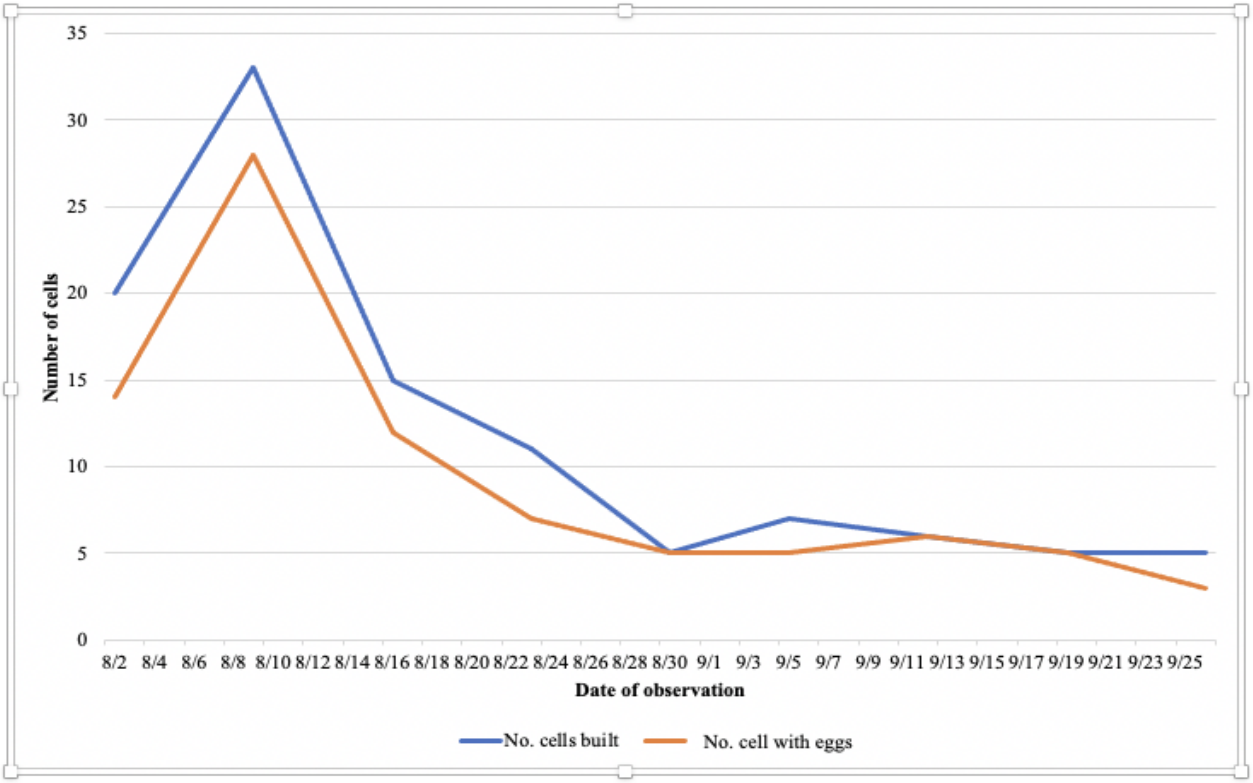

Figure 3. Dynamics of oviposition

cells built on August 9th was 33, which was the highest number during the duration of the experiment, and the recorded temperature was 25°C. However, there was a significant decrease in that number after nine days when the temperature increased to 35°C. Since then, the rate of built cells decreased to five on September 26th. Interestingly, fanning activities were recorded on several high-temperature days, similar to the report of Vollet-Neto *et al.* (2015). One of the most outstanding features was that the total number of egg-laying cells was always lower than the number of built cells. There were just three times that workers discharged food into all the built cells. These times were on August 30th, September 13th, and September 20th, 2019.

Conclusions

The stingless bee *Tetrigonilla collina* in the North of Vietnam had the following biological characteristics: the worker cell dimensions were 5.30 ± 0.34mm in length by 4.28 ± 0.37mm in width with a volume of 45.02 ± 5.74µL (food volume: 19.05 ± 3.5µL; ratio: 42.27%). The new cells were mostly built in the morning (from 2:00 am to 10:00 am) and the queen laid eggs (2.52 ± 0.81 sec per egg) and capped the cells in the afternoon (12:00 pm to 5:00 pm). The average worker-cell building period was 12.03 ± 3.78 hours. Mainly, six of first ten bees who inserted made food discharges in a cell. The food discharge occurred in very short time, lasting only 1.58 ± 0.60 minutes. The colony reduced the number of new cells made each day at the end of experimental period.

Acknowledgments

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